

ENERGY-PRODUCING ABILITY OF BACTERIA UNDER OXIDATIVE STRESS

by

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Abstract

Nitrosative stress is caused by reactive nitrogen species (RNS) and is toxic to most organisms. RNS are generated by the immune system to combat infectious microbes and are known to impede O₂-dependent energy production. The goal of this study was to elucidate alternative adenosine triphosphate (ATP)-forming pathways that enable the model bacterium *Pseudomonas fluorescens* to survive a nitrosative challenge in a fumarate medium. Fumarate was metabolized by fumarase C (FUM C), a RNS-resistant enzyme and fumarate reductase (FRD). The enhanced activities of pyruvate phosphate dikinase (PPDK), adenylated kinase (AK) and nucleoside diphosphate kinase (NDPK) provided an effective route to ATP production by substrate-level phosphorylation (SLP), a process that does not require O₂. The metabolic networks utilized to neutralize nitrosative stress reveal potential target against RNS-tolerant bacteria and a route to the conversion of fumarate into succinate, a value-added product.

Keywords

Reactive nitrogen species (RNS), *Pseudomonas fluorescens*, fumarate, ATP, substrate-level phosphorylation.

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Abbreviations

μM	Micromolar
μL	Microlitre
μg	Microgram
ACN.....	Aconitase
ADP.....	Adenosine diphosphate
AK.....	Adenylate kinase
APS.....	Ammonium persulfate
ATP.....	Adenosine triphosphate
AU.....	Absorbance units
BN.....	Blue native
BN-PAGE.....	Blue native polyacrylamide gel electrophoresis
BSA.....	Bovine serum albumin
C.....	Celsius
CFE.....	Cell free extract
cm.....	Centimeters
CoA.....	Coenzyme A
CO-IP.....	Co-immunoprecipitation
CSB.....	Cell storage buffer
DCIP.....	Dichloroindophenol
ddH ₂ O.....	Deionized and distilled water
ETC.....	Electron transport chain

FAD.....	Flavin adenine dinucleotide (oxidized)
FADH ₂	Flavin adenine dinucleotide (reduced)
Fe.....	Iron
FUM.....	Fumarase
FRD.....	Fumarate Reductase
GSNO.....	S-nitrosylated glutathione
GSNOR.....	S-nitrosylated glutathione reductase
h.....	hours
HCl.....	Hydrochloric acid
HPLC.....	High performance liquid chromatography
INT.....	Iodonitrotetrazolium
KDa.....	Kilodaltons
LDH.....	Lactate dehydrogenase
M.....	Molar
ME.....	Malic enzyme
MDH.....	Malate dehydrogenase
min.....	Minutes
mL.....	Millilitre
mM.....	Milimolar
mm.....	Millimeters
N.....	Normal (normality)
nm.....	Nanometers
NaCl.....	Sodium chloride

NAD.....	Nicotinamide adenine dinucleotide (oxidized)
NADH.....	Nicotinamide adenine dinucleotide (reduced)
NADP.....	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH.....	Nicotinamide adenine dinucleotide phosphate (reduced)
NAD-ICDH.....	NAD-dependent isocitrate dehydrogenase
NADP-ICDH.....	NADP-dependent isocitrate dehydrogenase
NDPK.....	Nucleotide diphosphate kinase
NO.....	Nitric oxide
NOS.....	Nitric oxide synthase
O ₂	Molecular oxygen
ONOO.....	Peroxynitrite
Pi.....	Inorganic phosphate
PPi.....	Pyrophosphate
PC.....	Pyruvate carboxylase
PEP.....	Phosphoenolpyruvate
PEPC.....	Phosphoenolpyruvate carboxylase
PMS.....	Phenazine methosulfate
PPDK.....	Pyruvate, phosphate dikinase
RNS.....	Reactive nitrogen species
ROS.....	Reactive oxygen species
sec.....	Seconds
SNP.....	Sodium nitroprusside
SOD.....	Superoxide dismutase

TCA.....	Tricarboxylic acid
TEMED.....	N,N,N,N –Tetramethylethylenediamine
v/v.....	Volume/volume
w/v.....	Weight/volume
WB.....	Western blo

Chapter 1: Introduction

1.1: Metabolism: The foundation of life

The foundation of life on this planet is a set of chemical reactions that take place within all organisms. These reactions are known as metabolism. The primary goal of metabolism is to sustain life in any given organism. The metabolic processes are involved in a variety of functions including in generating new cells, in the defense against other organisms, in repairing damaged cells, in communicating intracellularly and extracellularly, in the transport of essential nutrients, in the elimination of toxic compounds, and in the production of energy (Figure 1). All these actions are closely regulated by a numerous pathways that often work in a large network to produce the desired outcome.

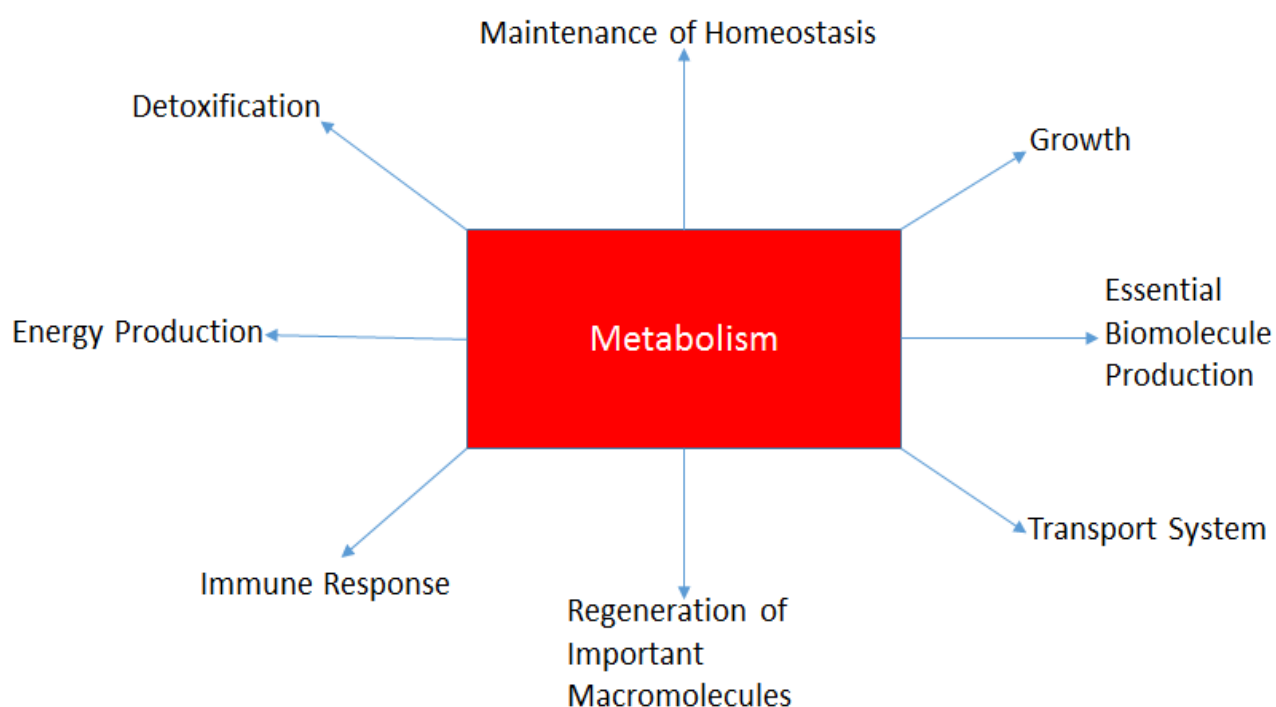


Figure 1.1: The important roles metabolism plays in a living system

All organisms, whether prokaryotic or eukaryotic utilize a source of carbon to maintain life (Wyatt et al 2014). Metabolism helps produce a variety of metabolites that contribute to the process of living. These metabolites are often used in the formation of carbohydrates, proteins, nucleic acids, lipids, and ATP, all of which are critical for the survival of any organism (Dalby et al 2011; Liu et al 2011; Nicholson et al 2012) (Figure 2).

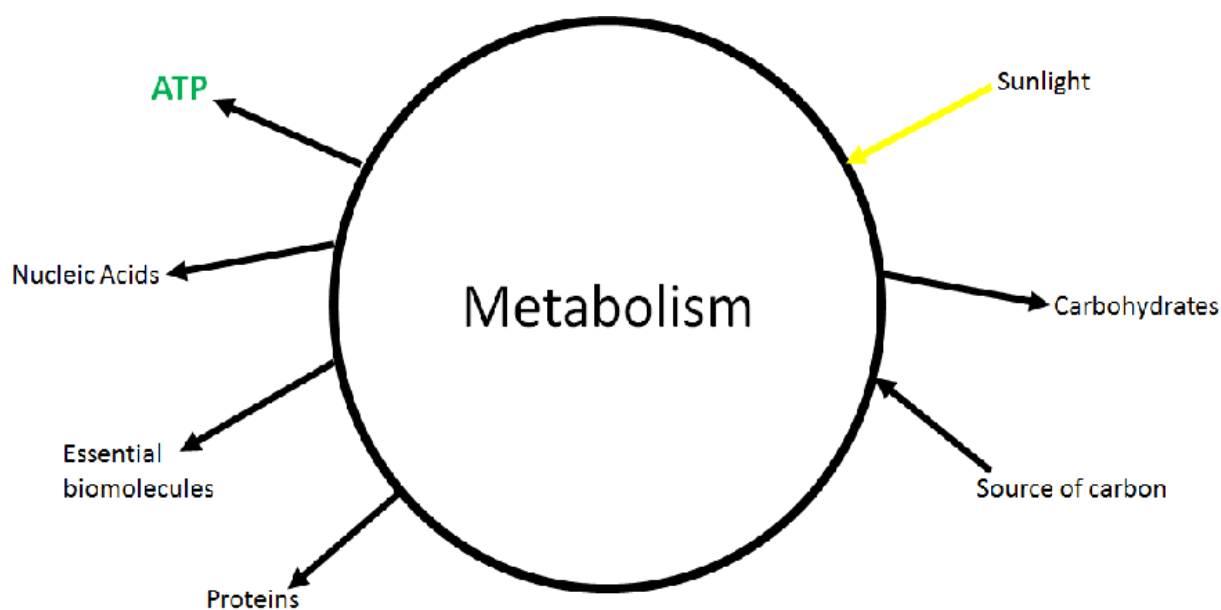


Figure 1.2: Various functions and actions of metabolism (Adapted from Dalby et al. 2011)

However, the most important function of metabolism is the production of energy. Without energy, there would be no life on this planet. Most biological energy comes in the form of adenosine triphosphate (ATP), a moiety where the energy is stored in the phosphodiester bonds. This biomolecule is the one that is used as the universal energy currency in both prokaryotic and eukaryotic cells. However, there are also other high energy phosphates that can be utilized as intermediates or as direct energy transporters instead of ATP. These include phosphoenolpyruvate (PEP), and 1,3-biphosphoglycerate (Baily et al 2011; Reddy and Wendisch 2014; Vander Heiden et al 2010). Both of these molecules have more stored energy content than ATP. They all contain phosphate moieties (Table I). This high energy phosphate bond is the key to biological energy, because when it is cleaved there is a release of energy into the system. The energy in ATP can also be transferred to other nucleotides like GTP, CTP, and UTP. There tends to be an equilibrium of these nucleoside triphosphates (Carraro et al 2014; Deramchia et al 2014; Selyunin et al 2011). Energy may also be stored in thioester bonds of acetyl and acyl coenzymes A.

Table I: List of high energy phosphates (Adapted from Carraro et al 2014)

<u>High Energy Phosphate</u>	<u>Mechanism for Production</u>
Adenosine triphosphate (ATP)	Common energy moiety in all living systems (oxidative phosphorylation)
1,3-biphosphoglycerate	Glycolysis (Glyceraldehyde phosphate dehydrogenase)
Phosphoenolpyruvate (PEP)	Glycolysis (Enolase)
Glucose-6-phosphate	Glycolysis (Hexokinase)
Guanosine triphosphate (GTP)	Successive phosphor-transfer from ATP
Uridine triphosphate (UTP)	Successive phosphor-transfer from ATP

1.2: Biological Energy-Producing Machines

Biological systems have evolved three distinct processes to generate ATP. Photophosphorylation is one of these mechanisms. As the name suggests, it is the process by which sunlight (photo-) is used by the cell to synthesize ATP (-phosphorylation). This form of energy production is found most commonly in organisms that possess a light harvesting apparatus. The light harnessing process can vary depending on the type of organism involved, such as the use of chlorophyll as the light absorbing molecule, as seen in most land plants, or the use of bacteriorhodopsin, a molecule that is also found in an altered form in our own eyes, that can be utilized to absorb the light. This is found most commonly in marine plants and bacteria (Skulachev et al 2013).

Following the absorption of light, the electrons stored in the H_2O molecules are released and transported to nictotinamide dinucleotide phosphate (NADP^+). The reduction of the latter generates NADPH, a critical reducing factor while the movement of the e^- , a process facilitated by the electron carriers helps create a proton gradient and a membrane potential. These are subsequently trapped as chemical energy in the form of ATP. Bacteriorhodopsin is both a light harvesting complex and a proton pump. Upon absorption of light, it produces a proton gradient that drives the synthesis of ATP (Figure 3) (Lanyi et al 2012; Skulachev et al 2013).

Photophosphorylation is mediated by a variety of factors. These most commonly take the form of internal inhibitors that are activated when energy needs are met in the organism. The ratio of $\text{NADP}^+/\text{NADPH}$ is a critical modulatory process. This electron transfer mechanism is used as a common means of producing ATP through photophosphorylation. Once the amount of NADPH exceeds the level of NADP^+ , normal ATP production is often slow down or halted. NADP^+ must be available as a reducing cofactor for the reaction and its decrease leads to a concomitant reduction of photophosphorylation. Additionally the buildup of NADPH triggers metabolic toxicity due to spontaneous reactions. As photophosphorylation is most commonly associated with plants, numerous studies have shown how artificial inhibitors can be introduced to inhibit normal energy production. One of the most common artificial inhibitors is 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU is a herbicide aimed at limiting the spread of invasive species of plants in the agricultural industry. DCMU acts on photosystem II by attaching itself to the plastoquinone binding site. This effectively blocks the flow of electrons from photosystem II to plastoquinone. Without this step, the flow of electrons is interrupted and the production of ATP is inhibited (Picard et al 2006).

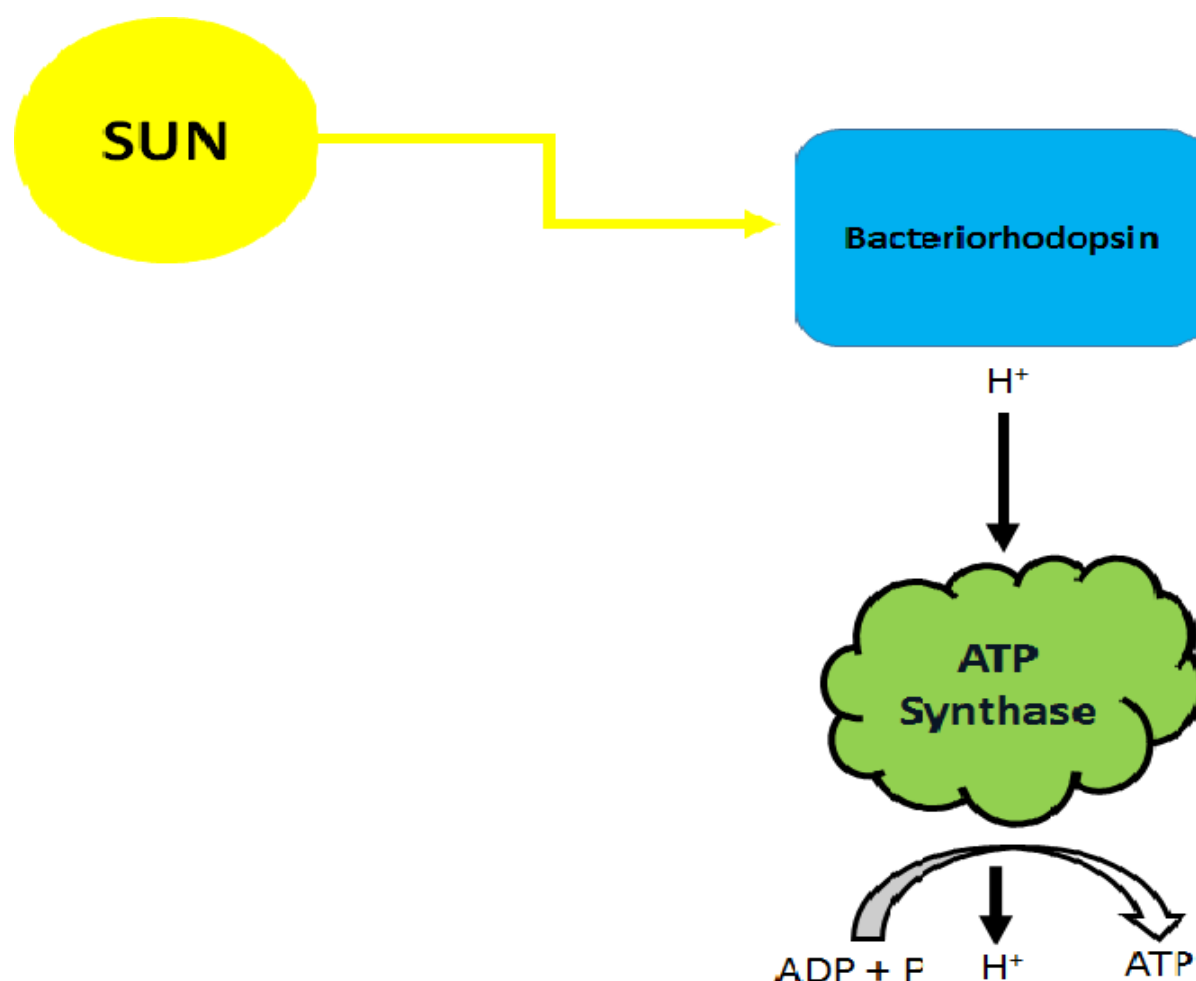


Figure 1.3: Action of bacteriorhodopsin in the production of ATP. Sun energy is absorbed by bacteriorhodopsin that generates a proton gradient (H^+). This in turn activates a transmembrane bound ATP synthase found in plants (Adapted from Picard et al 2006).

1.3: Oxidative Phosphorylation

Oxidative phosphorylation is the process utilized by aerobic organisms to generate energy. This process involves the oxidation of various biomolecules in order to form ATP. It is the widely utilized form of energy production in most non-photosynthetic life forms, due to the fact that it tends to be the mechanism with the highest eventual yield in energy. In most prokaryotic and eukaryotic systems, oxidative phosphorylation occurs following the generation of reducing factors from carbon sources (Besterio et al 2002; Brigaud et al 2006).

These reducing factors are generated in a metabolic module referred to as the tricarboxylic acid cycle (TCA cycle). Following the release of CO_2 , reduced nicotinamide dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH_2) are shunted from this system to release hydrogen ions (H^+) and e^- that are trapped within these moieties. The NAD^+ and the FAD^+ are subsequently returned to the TCA cycle to continue shunting reducing power from the system. The electron transport chain acts as a conduit to transport e^- and to form a charge gradient along a membrane, usually the mitochondria (Ritov et al 2010) in eukaryotes and the cytoplasmic membrane in prokaryotes (Biegel et al 2011). The movement of H^+ through complex V (ATP synthase) drives the synthesis of ATP. Through a mechanical action, this enzyme combines a phosphate molecule with a free ADP molecule to form ATP. The reduction of oxygen helps produce H_2O , a by-product of oxidative phosphorylation. Although this metabolic arrangement generates the most energy, this process is associated with a few drawbacks (Akram, 2013; Fernie et al 2004; Satapati et al 2012).

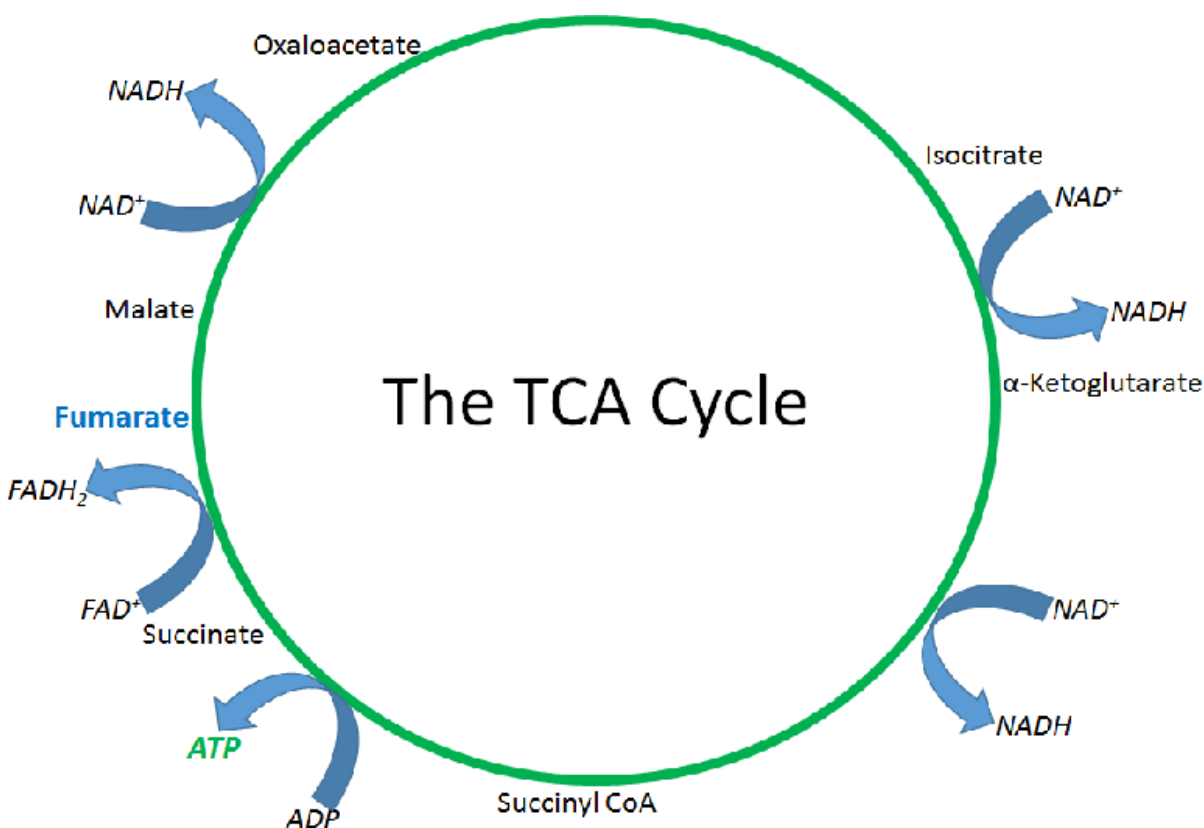


Figure 1.4: General TCA cycles and significant products produced throughout process.

Note the presence of fumarate and the formation of ATP (Adapted from Akram 2013).

The overall process requires the input of oxygen in order to safely remove the H^+ from the system. If not removed properly, the H^+ could stay in the system, thus destabilizing the gradient in the membrane, causing damage to the membrane and other systems in the organism. It is for this reason that these steps are often called aerobic respiration. Despite the fact that the TCA cycle does not need oxygen directly, it too shuts down in the absence of oxygen. This is due to the fact that without oxygen NADH and FADH₂ cannot be shunted to the electron transport chain. The buildup of these two biomolecules can prove to be even more dangerous as

the movement of e^- may be halted leading to the leak of this reactive species. The e^- can react to generate toxic moieties. Indeed the production of reactive oxygen species (ROS) is a major disadvantage to this high ATP yielding machine (Figure 5) (Celedon and Cline 2013; Sevilla et al 2013).

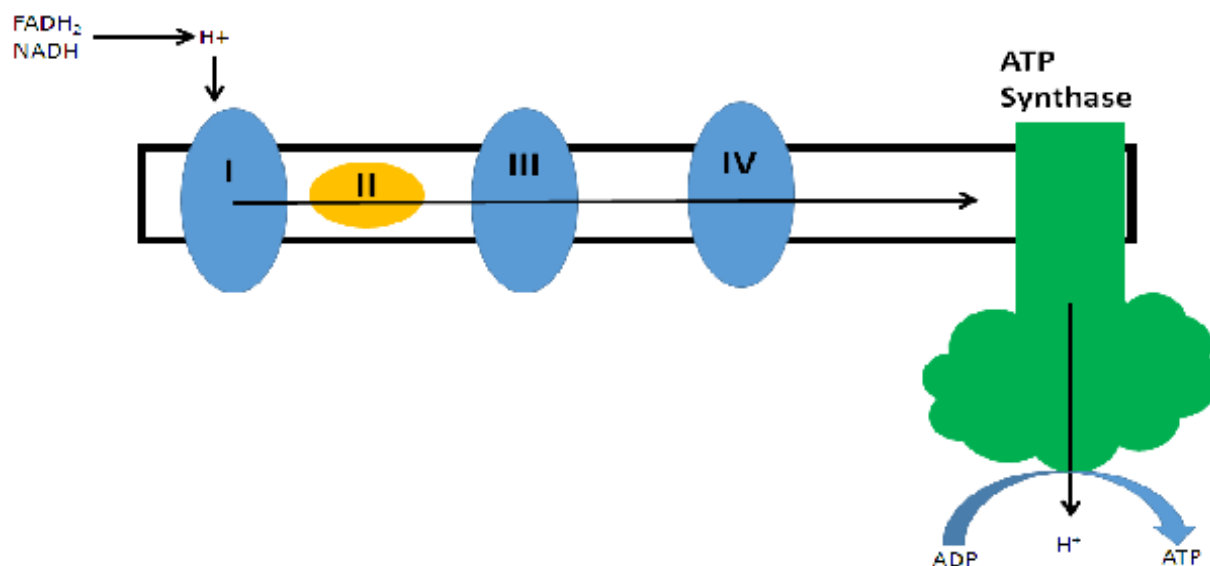


Figure 1.5: General outline of the electron transport chain (ETC) (Complexes I, II, III, and IV). Note the production of ATP through the gradient produced by the presence of H^+ (Adapted from Sevilla et al 2013).

As with the case of photophosphorylation, oxidative phosphorylation is regulated by various internal and external factors. The primary internal factor that controls energy production through this mechanism is the presence of reduced cofactors. $NADH$ and $FADH_2$ are used to transport electron from the TCA cycle to the ETC where they are used to produce ATP. As in the

case of NADPH in photophosphorylation, NAD^+/NADH and $\text{FAD}^+/\text{FADH}_2$ levels are essential to keep the energy producing machine operational. Buildup of the reduced cofactors cause toxicity within the metabolic system, as well as limiting the use of the unreduced form of these cofactors to propel the various dehydrogenases. Under these conditions, ATP production is often extremely limited, with the reduced cofactors diverted to the production of fatty acids and other biomolecules (Haq et al 2013; Mishra et al 2014).

The presence or lack of magnesium is also very important for any type of oxygen-dependent energy producing machinery. Magnesium in most living systems is used as a stabilizing element, specifically for compounds containing phosphates. Magnesium ions (Mg^{2+}) are used in biological systems to stabilize nucleotides in their triphosphate form. This means that in order for ATP to be maintained, produced, and released in the body, magnesium must be present. Most enzymes in oxidative phosphorylation contain magnesium, and Mg^{2+} plays a key role in the stabilization of ATP molecules. Hence, a deficiency of magnesium, can seriously inhibit the production of ATP during oxidative phosphorylation (Agarwal et al 2012; Yao et al 2011).

The presence of oxygen is also an important mediator of oxidative phosphorylation. Oxygen is pivotal to the production of energy via oxidative phosphorylation. It is the terminal molecule, and without its ability to trap e^- , there would be no ATP formation. A lack of oxygen will completely shut down normal oxidative phosphorylation. Oxygen can be depleted for a number of reasons, the most common of which is physical activity. During strenuous exercise, oxygen is utilized rapidly in order to supply energy to the muscles in motion. Due to this lack of oxygen, ATP cannot be produced at its normal level as an organism has to rely on alternate pathways like substrate-level phosphorylation (Bailey-Serres et al 2012; Pike et al 2011).

1.4: Substrate Level Phosphorylation

Substrate-level phosphorylation is also an important contributor to the ATP budget in all organisms. This strategy involves the direct transfer of a phosphate groups from a high energy metabolite to adenosine diphosphate (ADP) to form ATP. One of the hallmarks of this form of metabolism is the fact that it does not rely on any co-factors in order to produce energy. In most prokaryotic and eukaryotic systems, this type of energy production is the beginning of a larger process of energy production known as cellular respiration (Thakker C et al 2011). The yield of ATP during substrate-level phosphorylation is relatively low as compared to oxidative or photo phosphorylation. However, in the absence of oxygen, this form of energy production becomes the primary source of ATP synthesis. The other drawback to this system is the harmful by-products like ethanol in microbes and lactic acid in humans(Hunt et al 2010; Sharma et al 2012).

Glycolysis is a key generator of ATP via substrate level phosphorylation. The formation of 1,3,biphosphoglycerate and phosphoenol pyruvate (PEP) enables the synthesis of ATP (Figure 6). Pyruvate kinase (PK) mediates the formation of ATP from PEP and is highly modulated by the levels of ADP. This enzyme is also controlled by phosphorylation and de-phosphorylation (Luo et al 2011). The breakdown of glucose during glycolysis generates 2 net ATP while the same monosaccharides produce numerous fold more ATP during oxidative phosphorylation (Flamholz et al 2013; Jiang et al 2012).

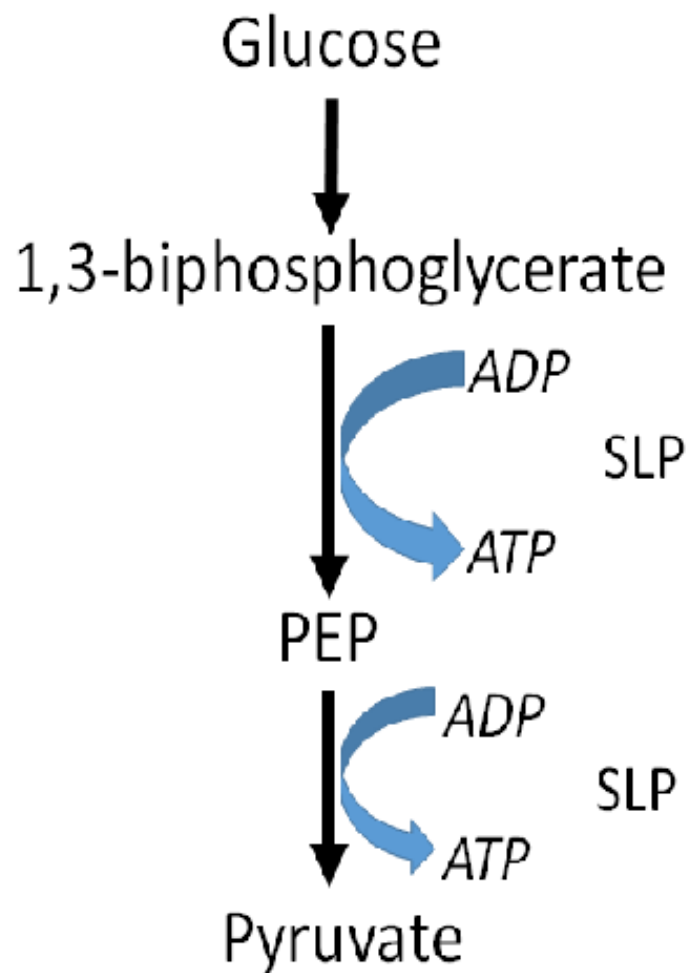


Figure 1.6: Glycolysis and ATP production. Substrate-level phosphorylation, an oxygen independent process, is the way in which energy is produced in this process (Adapted from Jiang et al 2012).

1.5: Damage of oxidative metabolism

While organisms do attempt to control metabolic activity as much as possible, these processes are highly complex, which often lead to unintended side-effects. These occur primarily during oxygen dependent forms of energy production. When an organism undergoes oxidative phosphorylation, electrons are transported through the ETC in order to generate ATP, and H_2O , with the help of O_2 . Due to the size and mobility of these electrons, there is a potential for electrons to escape, or “leak”, out of the ETC. These electrons can then react with the oxygen in the system in the system to produce superoxide radical ion ($\text{O}_2^{\cdot-}$). This compound is first in a series of highly reactive compounds known as reactive oxygen species (ROS). Through a series of reactions with the superoxide radical ion, electrons, and the redox reaction of Fe^{3+} to Fe^{2+} , more unstable oxygen-based molecules are produced in the system. These highly reactive species can cause serious toxic effects in an organism. Hence it is not surprising that all aerobic organisms have evolved intricate strategies to neutralize these toxic species as they are constantly bombarded by ROS. Catalase, superoxide dismutase, and glutathione peroxidase are some ROS-combatting enzymes that are prominent in numerous aerobes (Murphy et al 2011; Naik and Dixit 2011).

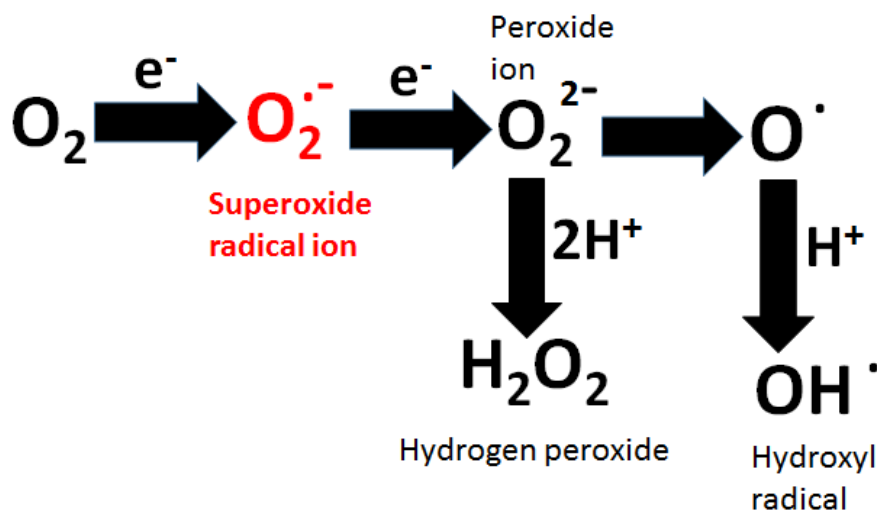


Figure 1.7: Production of harmful ROS in living systems. Note the production of the superoxide radical ion (Adapted from Murphy et al 2011).

1.6: Reactive Nitrogen Species (RNS)

Reactive nitrogen species (RNS) are another group of transient moieties that are involved in numerous biological processes. Initially, the study of these molecules has been focused on their positive effects in living systems. RNS are often intentionally produced by an organism through reactions with the amino acid arginine and the enzyme nitric oxide synthase. This reaction yields citrulline and nitric oxide (NO). The primary reason that these potentially hazardous molecules are intentionally produced by an organism is to participate in signaling duties (Michaelson et al 2013; del Rio 2011). Due to the highly reactive nature of NO and its derivatives, they make effective initial signals in a biochemical signalling pathway. An example of this action is during mitochondrial functions, NO is often the first signal in the mitochondrial metabolic network. Intentionally produced RNS are often separated into three distinct groups;

neuronal RNS (nRNS), endothelial RNS (eRNS), and inducible RNS (iRNS). As each name suggests, each RNS molecule is used in a specific signalling network involving neuronal activity, endothelial signalling, and to induce an action involved in signalling, respectively (Mak et al 2006; Ward et al 2013). While nRNS and eRNS are often continually produced, due to the necessity of neuronal and endothelial action for maintaining life, this is not the case with iRNS. This iRNS network has to be induced in order to produce NO. The reason for this is that iRNS is used only in the case of defense, and operates independently of Ca^{2+} , an essential mechanism is the production nRNS and eRNS. NO is a highly volatile substance which can be effectively used in defense against bacterial threats. Numerous organisms use RNS for a variety of essential functions like seed germination, neuron activity, and cardiac stimulation (Graves 2012; Salvemini et al 2011).

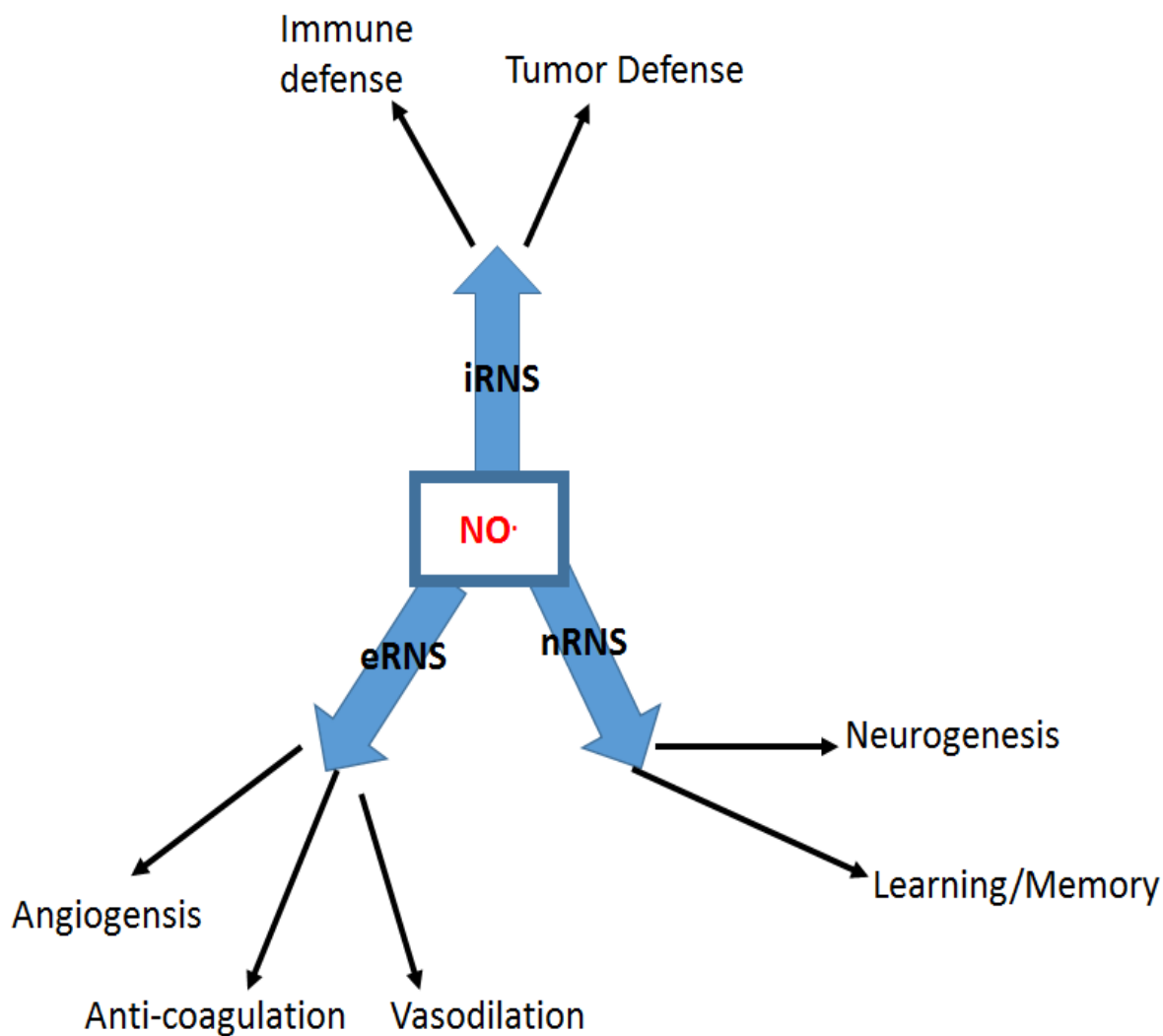


Figure 1.8: Nitric oxide and its various biological functions (Adapted from Graves 2012)

1.7: Uncontrolled Production of RNS

ROS can produce direct effects on macromolecules but they are also the primary means by which toxic RNS are generated. Most reactions involving macromolecules (lipids, proteins,

etc.) and ROS cause the production of free radicals in the affected macromolecules. For example, when an ROS species comes in contact with a lipid chain and rapid reaction occurs usually with the labile double bond. The result of which is endogenous oxygen, or other less harmful by-product, and a lipid molecule containing a free radical. This free-radical can now react with other surrounding lipid chains, thus destroying the integrity of a molecular subunit, like a phospholipid bilayer (Bourret et al 2011; Wang et al 2013).

The principles by which these reactions occur can be extended to the production of RNS. When an ROS compound reacts with a free nitric oxide, often produced effect a biological function, it will undergo a spontaneous reaction, due to the highly reactive state of both compounds. This initial reaction results in the production of peroxynitrite (ONOO^-). This is a critical molecule in RNS toxicity due to its highly reactive nature. While ROS molecules often react with the macromolecule in come in contact with, peroxynitrite reaction often occur at a much slower rate. While this means there is a higher chance of detoxification, it also means the molecule can be far more selective, meaning it can attack targets that could be a weaker and far more essential. This moiety also has the ability to pass through cellular membranes, something that some ROS species cannot do readily. Hence, both the external and internal environments of the cell are susceptible to attack. Peroxynitrite can undergo further reactions with other molecules to produce other high reactive species such as nitrogen dioxide ($-\text{NO}_2$), dinitrogen trioxide (N_2O_3), as well as other RNS molecules (Hayashi et al 2012; Vitteta and Linnane 2014).

1.8: Toxic effects of RNS

RNS molecules are involved in similar destructive reactions like ROS. These include oxidation of lipids, proteins and nucleic acids. In all these cases a free radical is produced on the

macromolecule, which causes them to propagate the abnormality to other surrounding molecules, or completely compromises the molecular system rendering them useless (Belechiev et al 2012; Corpas and Barroso 2013). Additionally peroxynitrite has a strong affinity to attack metal centers in enzymatic proteins. These centers are often essential for a particular enzymatic activity, which are often involved in redox reactions. Most of these enzymes contain iron, an important metal utilized during a variety of processes like oxidative phosphorylation. This renders essential function ineffective. RNS and ROS have been linked to disorders related to aging and to the cerebral system (Williams et al 2008).

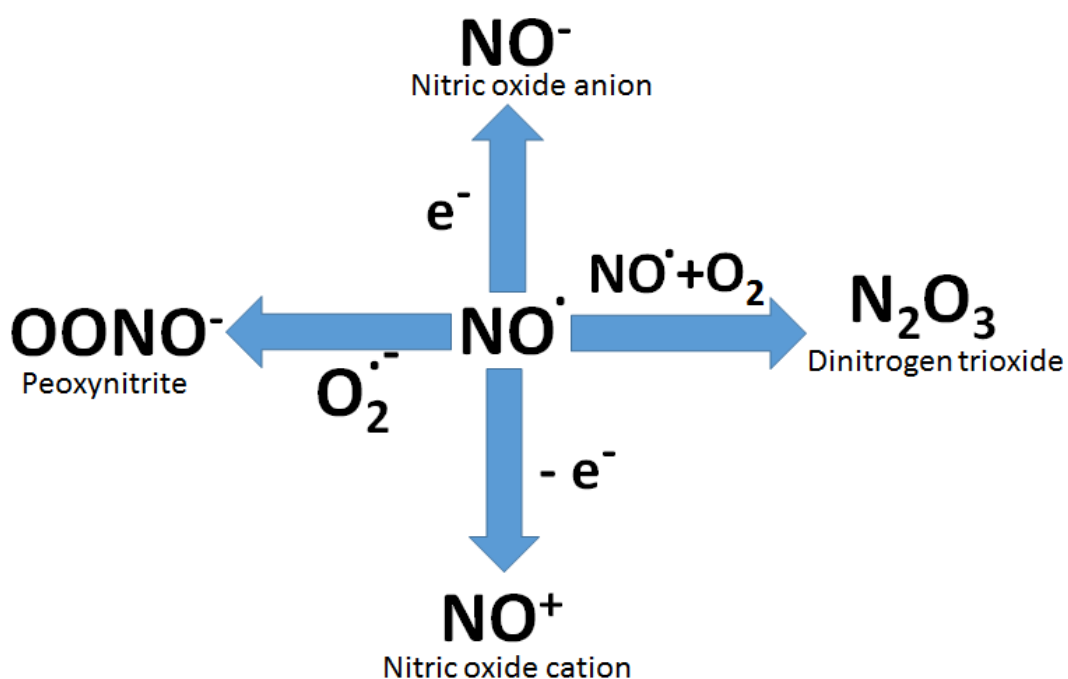


Figure 1.9: RNS creation and propagation causing toxic effects (Adapted from Belechiev et al 2012).

1.9: Traditional defenses to RNS

As RNS are generated in living systems, numerous organisms have evolved various strategies to counter these toxic compounds. Their conversion into nitrates and nitrites by nitrate and nitrite reductase respectively tend to make them less toxic. In humans, myoglobin is known to be a detoxifier of NO (Hu et al 2012). Some bacteria have been shown to invoke flavohemoglobin to eliminate RNS. The ability of s-nitroso glutathione reductase (GSNOR) to promote denitrosylation is also widely utilized to detoxify biomolecules compromised by RNS. Additionally NADPH producing enzymes are quite effective at curtailing the formation. Hence, malic enzyme and isocitrate dehydrogenase-NADP⁺ dependent are upregulated during nitrosative stress (Alvarez et al 2013).

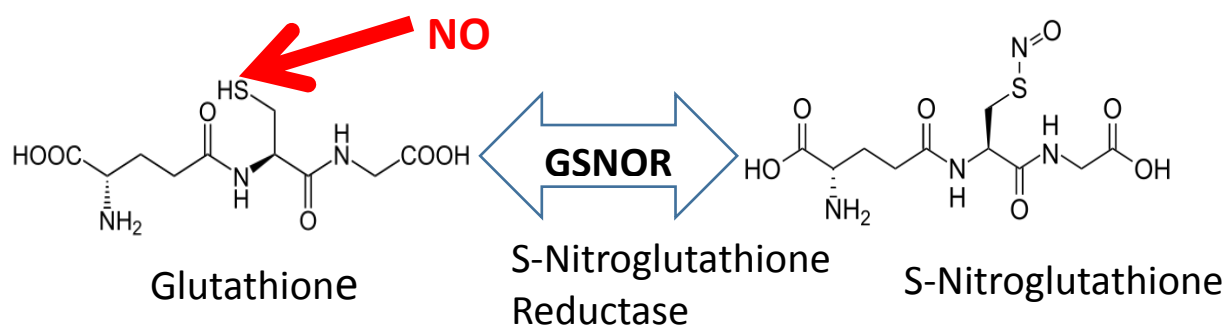


Figure 1.10: Action of GSNOR in detoxifying RNS. Note this reaction can also be reversible to produce NO for signalling (Adapted from Hu et al 2012).

1.10: Fumarate metabolism and *Pseudomonas fluorescens*

During oxidative phosphorylation, glucose, the six-carbon monosaccharide is broken down in a series of chemical reactions. Throughout this process, glucose is transformed into numerous smaller moieties. Of particular interest are the four-carbon products. These molecules help propel the TCA cycle, and are often involved in the direct production of ATP. One of these is fumarate. Fumarate is a four carbon dicarboxylic acid that contains a double bond. During the TCA cycle, fumarate is generated from succinate a reaction mediated by the enzyme succinate dehydrogenase (Raimundo et al 2011). It can also be produced with malate via the enzyme fumarase. This hydration reaction is important for the reconstruction of initial metabolites used in the TCA cycle that will be needed again to produce ATP. It is also produced during the degradation of nucleic acid. Numerous commercial applications have been found for fumarate. These include manufacture of polyester products, an initial molecule used in the synthesis of polyols (the key ingredient of artificial sweeteners), and in commercial dyes. Recent studies have also suggested application in some natural health treatments. However, fumarate can also be an important source of succinate and malate metabolites that are also of immense economic values in biopolymers such as polybutylene succinate and polyacetic acids, for foods and pharmaceutical industries (Luo et al 2010; McKinlay et al 2010).

The ability of organisms to effectively produce ATP is critical for their survival. Any perturbation in this process may lead to their demise. Although RNS toxicity is known to inhibit oxidative phosphorylation, some organisms do proliferate under nitrosative stress (Lushchak 2011; Voskull et al 2011). Hence, alternate ATP generating mechanisms must be operative for these organisms to multiply. While nutrient such as citrate and glucose have been shown to be rapidly metabolized in environments with nitrosative stress, the ability of microbes to metabolize

fumarate, a key ingredient in the TCA cycle has yet to be studied (Chabriere et al 1999; Garchowski et al 2012; Trapani et al 2001). Additionally, the enzyme fumarase involved in the degradation of fumarate is an Fe-dependent moiety that is known to be seriously affected by RNS.

Pseudomonas fluorescens, a soil microbe is an excellent model system to study these processes as it grows rapidly and is nutritionally versatile. Its metabolism has also been shown to be highly flexible; it can survive stressing agents, and adverse environments. Furthermore, *P. fluorescens* is the same genus as *Pseudomonas aeruginosa* as they share numerous common metabolic networks. The latter microbe has been linked to pneumonia, urinary tract infections, and necrosis of the skin. Recent studies have found that some strains of *P. aeruginosa* have become resistant to traditional antibiotic treatments (Omri et al 2002; Suntres et al 2002). Hence, it is quite likely that the adaptive mechanisms elucidated by *P. fluorescens* may have some similarities to infectious bacteria such as *P. aeruginosa*. The discovery of novel pathways to generate ATP and to metabolize fumarate under nitrosative stress may unveil targets against antibiotic resistant bacteria and provide value-added products (Chenier et al 2008).

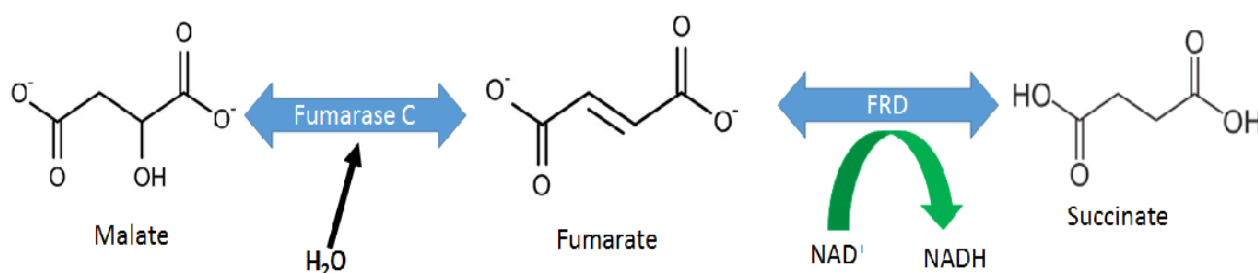


Figure 1.11: Fumarate and its reactions in the TCA cycle (Adapted from Raimundo et al 2011).

1.11 Research Objectives

The goal of this study was to evaluate the energy-producing capabilities of bacteria under the influence of RNS stress. This was carried out by probing substrate-level phosphorylating enzymes, including those that are not utilized under normal conditions. These alternate energy pathways are often used by infectious bacteria to circumvent host immune systems and antibiotics. If the enzymes in these alternate ATP-generating modules are found to be absent in the host, they may be an ideal target to eliminate these bacteria with minimal side-effects on the host. Additionally the ability of the microbe to utilize fumarate as its carbon source was evaluated, particularly under RNS-challenged conditions. The four-carbon molecule is usually metabolized via the TCA cycle, a metabolic network that is known to be severely impeded under nitrosative stress. The conversion of fumarate into value-added products like succinate would be of important economic benefit. This saturated dicarboxylic acid has a wide range of commercial applications.

Chapter 2

Fumarate metabolism and ATP production in *Pseudomonas fluorescens* exposed to nitrosative stress

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2.1: Abstract

Although nitrosative stress is known to severely impede the ability of living systems to generate adenosine triphosphate (ATP) via oxidative phosphorylation, there is limited information on how microorganisms fulfill their energy needs in order to survive reactive nitrogen species (RNS). In this study we demonstrate an elaborate strategy involving substrate-level phosphorylation that enables the soil microbe *Pseudomonas fluorescens* to synthesize ATP in a defined medium with fumarate as the sole carbon source. The enhanced activities of such enzymes as phosphoenolpyruvate carboxylase (PEPC) and pyruvate phosphate dikinase (PPDK) coupled with the increased activities of phospho-transfer enzymes like adenylate kinase (AK) and nucleoside diphosphate kinase (NDPK) provide an effective strategy to produce high energy nucleosides in an O₂-independent manner. The alternate ATP producing machinery is fuelled by the precursors derived from fumarate with the aid of fumarase C (FumC) and fumarate reductase (FRD). This metabolic reconfiguration is key to the survival of *P. fluorescens* and reveals potential targets against RNS-resistant organisms.

2.2: Introduction

Nitrosative stress occurs due to the uncontrolled formation of such reactive nitrogen species (RNS) as nitric oxide (NO), dinitrogen trioxide (N₂O₃), and peroxynitrite (ONOO⁻). These RNS are generated intracellularly when NO reacts with hydrogen peroxide and superoxide. They are known to be toxic as these moieties react with sulphydryl groups, redox metals, heme residues and tyrosine-containing macromolecules (Quijano et al 1997; Zielonka et al 2012). Hence, nitrosative stress is known to severely impair oxidative phosphorylation (OP), a

process that produces ATP in an O₂-dependent manner. This ATP-generating system relies on the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). While the former provides the reducing factors NADH and FADH₂, the latter aids in the shuttling of electrons to O₂, the terminal electron acceptor (Lemire et al 2012). Numerous enzymes that participate in these metabolic networks require heme and Fe-S clusters for proper functioning and are rendered ineffective by RNS (Auger et al 2011; Poole, 2005). Hence it is not surprising that numerous microbes have developed mechanisms to deal with these toxic RNS. Their conversion into innocuous nitrate, and the upregulation of enzymes dedicated to the removal of nitrosylated moieties, a common occurrence during nitrosative stress, are two strategies invoked by some bacteria (Auger et al. 2011). Although the detoxification mechanisms aimed at RNS have been studied, there is a dearth of information on how RNS-tolerant microbes satisfy their ATP requirements.

Substrate level phosphorylation (SLP) is the other ATP-producing machine that is commonly utilized by biological systems. High energy compounds that are obtained following various biochemical transformations help phosphorylate adenosine monophosphate (AMP) and/or adenosine triphosphate (ADP) into ATP (Kim et al. 2013). These compounds include phosphoenolpyruvate (PEP) and 1,3-bisphosphoglycerate, that are products of glycolysis, as well as succinyl CoA which is produced during the TCA cycle (Han et al. 2013; Singh et al 2009). Pyruvate kinase is a key enzyme that converts PEP into ATP in the presence of ADP (Auger et al 2012). Some bacteria are also known to possess the acetate kinase-phosphotransacetylase system that is involved in the processing of acetyl CoA into ATP (Hunt et al 2010; Ingram-Smith et al 2006). Although ATP formation mediated by SLP is widespread in nature, the metabolic

networks that are utilized to fulfill the need for this universal energy currency under environmental stress along with its regulation have yet to be fully elucidated.

As part of our efforts to decipher the metabolic adaptations that allow the soil microbe *Pseudomonas fluorescens* to survive in extreme environments (Auger et al 2011), we have evaluated how this nutritionally-versatile microbe fulfills its requirements for ATP, under a nitrosative challenge. This condition is known to render oxidative phosphorylation ineffective. In this study fumarate a TCA cycle intermediary and a dicarboxylic acid that is usually metabolized by RNS-sensitive Fe-S cluster rich enzymes was utilized as the sole source of carbon. The ability of *P. fluorescens* to elaborate an alternate-ATP generating network and the metabolism of fumarate are described. The significance of these findings in combatting RNS-resistant microbes is also discussed.

2.3: Materials and Methods

Bacterial Growth Conditions

P. fluorescens, strain ATCC 13525, was grown in a phosphate growth medium containing Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (0.8 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), and fumarate (2.25 g) per 1 liter of distilled and deionized water. One mL of trace elements was added per liter of medium as described in Bignucolo et al. (2013). Nitrosative stress was achieved with the addition of sodium nitroprusside (SNP) at a concentration of 10 mM (Auger et al 2011). Cultures pH was adjusted to 6.8 using dilute NaOH. Previous studies have shown that *P. fluorescens* has the ability to reach its optimal growth at this pH (Auger et al 2011). The mixtures were then transferred to 500 mL Erlenmeyer flasks. Each flask was filled with 200 mL of medium.

Cultures were inoculated by adding 1 mL of stationary phase bacteria to the growth media. These cultures were aerated on a gyrator water bath shaker (Model 76, New Brunswick Scientific). Once the stationary phase was reached, portions of samples were centrifuged at 10000 x g for 10 min at 4°C. Aliquots (10 mL) of spent fluid were stored for further experiments. Washing of the bacterial pellet was then completed using 0.85% NaCl, and the pellet was re-suspended using a cell storage buffer (50 mM Tris-HCl, 5 mM MgCl₂, and 1 mM phenylmethylsulphonyl fluoride, pH 7.3). Membranous and soluble cellular fractions were obtained by sonication and centrifugation of the disrupted cells (al-Aoukaty et al 1992). Bradford assays were performed on both fractions in triplicate to determine protein concentration. These fractions were kept at 4°C for 5 days or at -20°C for 4 weeks for further study.

RNS-Detoxifying Enzymes and Functional Metabolomic Studies

To evaluate the impact of the nitrosative stress on the bacterium, cultures were grown in control and nitrosative stressed-conditions at various time intervals and cellular yield was assessed by Bradford assay (Bradford 1976). Fumarate consumption was monitored by high performance liquid chromatography (HPLC) at a wavelength of 236 nm (Charoo et al. 2014). The presence of nitrate reductase and nitrite reductase were also probed to determine the relative activity of these detoxifying enzymes in each system. In gel activity of the enzymes was monitored and densitometric readings were recorded to obtain relative activities (Auger et al 2010). The Griess assay was performed on the spent fluids from both control and RNS-stressed cultures in order to determine relative content of nitrate and nitrite respectively (Miranda et al. 2001).

To assess significant metabolite level differences in control and RNS-exposed cultures, HPLC was performed on the spent fluids and soluble fractions of. Two mg/mL of protein equivalent of the cell cell-free extract (CFE) were taken and heated gently to ensure precipitation of proteins and lipids (Mailloux et al. 2011). The supernatants were subsequently monitored at 210 nm and 254 nm respectively. All metabolite levels were compared to known compounds, and by spiking with the appropriate standards (Auger et al. 2011).

Analyses of TCA Cycle and ETC Enzymes

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed to detect relative activity of enzymes in control and stressed cultures involved in the TCA cycle and in the electron transport chain (ETC). Proteins were prepared for BN-PAGE by dilution with native buffer (50 mM Bis-Tris, 500 mM ϵ -aminocaproic acid, pH 7.0, 4°C) until proteins were at a concentration of 4 mg/ml. One % (v/v) β -dodecyl-D-maltoside was added to the membranous fractions to help in the solubilization of the proteins. Once the gel had finished separation, it was placed in a reaction buffer (25 mM Tris-HCl, 5 mM MgCl₂, at pH 7.4) for 15 minutes to rinse off buffers. Once completed, specific protein fractions were separated and placed in their appropriate reaction mixtures where enzyme reactions were performed. All reactions were based on the presence of a pink formazan precipitate, formed during reduction reactions (Schrager and von Jagow 1991; Han et al 2012).

NAD⁺-dependent isocitrate dehydrogenase (ICDH-NAD⁺) activity was visualized using a reaction mixture containing 5 mM isocitrate, 0.5 mM NAD⁺, 0.2 mg/mL PMS and 0.4 mg/mL INT. For malate dehydrogenase (MDH) malate was used as the substrate for the reaction. Complex II was detected by the addition of 0.5 mM FAD⁺, and 0.4 mg/mL INT with 5 mM

succinate. Complex IV was probed using diaminobenzidine (10 mg/mL), cytochrome C (10 mg/mL), sucrose (562.5 mg/mL). In the case of both ETC complexes probed, the reaction were made in reaction buffer with the addition of 5mM of KCN. Appropriate negative controls consisted of reaction mixtures that did not contain the substrate or cofactors for the reaction. For example, reaction mixtures devoid of isocitrate and/or NAD^+ were utilized as negative controls for ICDH- NAD^+ while the commercial enzyme was tested as a positive control. Once completed all of the above reactions were destained using 40% methanol, 10% glacial acetic acid. Activity bands were quantified using ImageJ for Windows. Proper protein loading was determined by Coomassie staining for total proteins. Equal amounts of proteins (60 μg) were loaded in the respective lanes. Following the appearance of the activity bands (at the same time), the bands were excised and incubated in the reaction mixture to monitor the products formed. Enzymes such as malate dehydrogenase (MDH) and pyruvate carboxylase (PC) that had similar activity in the control and stressed cultures were utilized as internal controls. Unless otherwise mentioned, all comparative experiments were performed at the late logarithmic phase of growth. Select activity bands were excised and incubated in the appropriate reaction mixtures. The substrates and products of these mixtures were detected by HPLC.

Fumarate Metabolism and ATP Synthesizing Enzymes

In an effort to evaluate how fumarate was metabolized the cell-free extract (2 mg/mL protein equivalent) was incubated with 2mM of fumarate in the presence or absence of KCN (5mM). The soluble cell free extract (2 mg/mL) was also incubated with 2 mM oxaloacetate, 1 mM PP_i , and 0.5 mM AMP in order to determine how ATP was being generated. These reactions were performed with and without KCN. The production of ATP and other metabolites was monitored by HPLC. Fumarase was identified by in-gel activity assay using a reaction mixture

containing 5 mM fumarate, 5 units/mL of malate dehydrogenase, 0.5 mM NAD^+ , 0.2 mg/mL PMS and 0.4 mg/mL INT. The two isoforms of fumarase A and C were detected with the aid of inhibitors as described in Chenier et al (2008). Fumarate reductase (FRD) was also probed using succinate and NAD^+ (Watanabe et al 2011). The activity of, pyruvate carboxylase (PC), was ascertained by utilizing enzyme-coupled assays as described (Singh et al. 2005). PPDK was monitored using a reaction mixture consisting of 5 mM PEP, 0.5 mM AMP, 0.5 mM sodium pyrophosphate (PP_i), 0.5 mM NADH, 10 units of LDH, 0.0167 mg/mL of DCIP and 0.4 mg/mL of INT. Adenylate kinase (AK) was detected by an enzyme-coupled assay involving hexokinase and glucose-6-phosphate dehydrogenase (G6PDH). The ability of the enzyme to convert ADP to ATP enabled the conversion of glucose into glucose-6-phosphate that was then detected by the precipitation of formazan in the gel. Nucleoside diphosphate kinase (NDPK) in gel assay, was performed as described in Singh et al (2006). These bands were excised and their activities were followed by HPLC. Spectrophotometric analyses were also performed to confirm the activities of some select enzymes. MDH and ICDH- NAD^+ dependent were monitored by following the formation of NADH at 340 nm in the CFE in the presence of their respective substrates (Auger et al 2011).

Data were expressed as \pm standard deviation. Statistical correlations and significance of data were all confirmed using the Student T test ($p \leq 0.05$). All experiments were performed at least twice and in triplicate.

2.4: Results and Discussion

When *P. fluorescens* was exposed to nitrosative stress in a defined phosphate medium with fumarate as the sole source of carbon, the biomass at the stationary phase of growth was similar to that of the control culture. Although growth rate was slower in the stressed cultures, fumarate was utilized at a faster rate (Fig. 1). The impact of nitrosative stress was evident in the stressed bacteria, as there was an increase of 18 and 13 fold in activity of nitrite and nitrate reductase respectively. The presence of elevated levels of nitrate and nitrite in the spent fluid indicated that RNS was also being detoxified (Table 1). The disparate metabolic profiles observed in the soluble CFE between control and stressed cultures at the same phase of growth indicated a shift in metabolic pathways. Peaks attributed to pyruvate, and phosphoenolpyruvate (PEP) and AMP were also more intense in the bacteria obtained from the RNS-exposed cultures. The control cultures had higher levels of ADP (Fig 1).

As there was a stark difference in these nucleoside phosphate levels, it became apparent that the ATP-producing machinery had been affected. Analysis of select TCA cycle enzymes and the electron transport chain revealed that ICDH-NAD⁺ dependent, Complex II and Complex IV decreased in RNS-exposed bacteria. MDH did not appear to change in the control and stressed cultures (Fig. 2). Indeed the activity of ICDH-NAD⁺ was about 3 fold higher in the control compared to the stressed cultures as measured spectrophotometrically. MDH activity was found to be similar in these two conditions (Table 1). Hence, it was important to discern how ATP was generated since the TCA cycle and ETC enzymes were ineffective in the stressed cultures. When the CFE of the control and stressed *P. fluorescens* were incubated with fumarate in the presence of KCN, a potent inhibitor of oxidative phosphorylation (OP), ATP production was severely decreased in the control. However, ATP levels in the CFE obtained from the RNS-challenged

cells remained relatively similar with and without the presence of KCN. This indicated that *P. fluorescens* was utilizing an alternate metabolic network, independent of OP, to generate ATP in an effort to combat the toxic influence of the nitrosative stress (Fig. 2).

Fumarate is usually metabolized by fumarase, an enzyme known to be sensitive to oxidative and nitrosative stress (Lushchak et al 2014,). In this instance, *P. fluorescens* upregulated the activity of fumarase C, an isoenzyme known to be less prone to nitrosative stress. Two bands were evident in the electrophoregram obtained from BN-PAGE analysis (Fig. 3). Fumarate reductase (FRD), another enzyme involved in fumarate metabolism was prominent in the stressed cells while the activity band attributable to this enzyme was barely discernable in the control cells (Fig. 3). Hence, Fum C and FRD were utilized to degrade fumarate in the stressed bacteria. The presence of elevated amounts of phosphoenol pyruvate (PEP) in the CFE of the stressed cultures, led us to analyze enzymes responsible for the production of this metabolite. PEPC, an enzyme known to mediate the transformation of oxaloacetate into PEP, was increased as was PPDK (Fig. 3). The former utilizes inorganic phosphate (P_i) while the latter invokes the participation of PEP, AMP and PP_i to form pyruvate and ATP. This metabolic arrangement would provide an effective means of producing ATP in an O_2 -independent manner. Indeed similar ATP-generating networks have been uncovered in infectious organisms (Adam 2001; Couston et al 2003; Hall and Ji, 2013; Ma et al 2013). However, it was important to evaluate the biochemical processes involved in the fixation of ATP and the formation of AMP, a feature critical for this energy-generating machinery to work efficiently via substrate-level phosphorylation.

Adenylate kinase (AK), an enzyme that orchestrates the synthesis of ATP from ADP produces AMP, while nucleoside diphosphate kinase (NDPK) is able to transfer the high energy

phosphate from ATP to NDP or dNDP. Indeed both of these enzymes were found to have enhanced activities in the culture obtained from the stressed media compared to the control (Fig. 4). When the activity band was excised and incubated with the respective substrate, the formation of ATP and AMP from ADP in the case of AK was evident. The excised activity band of NDPK readily gave peaks indicative of ATP and GDP when incubated with ADP and GTP (Fig. 4). To confirm the workings of this metabolic network oxaloacetate, a product of fumarate, AMP, and PP_i were incubated with the soluble CFE from stressed and control bacteria obtained at the same phase of growth. Peaks attributed to PEP, GTP, and ATP, in the CFE isolated from the stressed *P. fluorescens* provided elegant evidence for the energy generating machine that was responsible for fuelling the survival of the stressed bacteria challenged by nitrosative stress.

Although the detoxification mechanisms involved in nitrosative stress have been subject to numerous investigations, the molecular pathways involved in the maintenance of ATP homeostasis under these conditions has yet to be fully unravelled. This study shows that *P. fluorescens* elaborates an intricate network to fulfill its energy needs. The pivotal role of the TCA cycle in generating ATP by substrate-level phosphorylation in *P. fluorescens* exposed to aluminum toxicity was recently demonstrated (Singh et al 2011). In this instance the upregulation of ICDH-NADP dependent and isocitrate lyase allows to combat the ineffectiveness of the Al-sensitive aconitase in order to generate glyoxylate and NADPH. The former then is transformed into oxalate and ATP, a process that is mediated by oxalate acetylating dehydrogenase, oxacyl CoA transferase and succinyl CoA synthetase. NADH and NADPH levels are also intricately modulated during stress (Mailloux et al, 2011; Li et al 2014). In this study, PPDK in tandem with the phosphotransfer systems involving AK and NDPK, provides an efficient route to ATP with the concomitant formation of AMP and NTP. Indeed, infectious

organisms that are subjected to the defense mechanisms of their host are known to invoke SLP, using PPK to produce ATP. Strategies to inhibit these enzymes may provide therapeutic cues against these microbes. In conclusion, findings in this report further reveal the nutritional versatility and adaptability of *P. fluorescens* and demonstrate the intricate metabolic network it utilizes to fulfill its requirements for ATP when subjected to nitrosative stress. The metabolic module may be a practical target to arrest infectious organisms known to invoke this pathway to thwart host defenses.

2.5: References

Adam RD (2001). Biology of *Giardia lamblia*. Clin Microbiol Rev. 14: 447-475.

al-Aoukaty A, Appanna, VD, and Falter H (1992) gallium toxicity and adaptation in *Pseudomonas fluorescens*. FEMS Microbiol Lett. 71: 265-272.

Auger C, Han S, Appanna VP, Thomas SC, Ulibarri G and Appanna VD (2012) Metabolic reengineering invoked by microbial systems to decontaminate aluminum: Implications in bioremediation technologies. *Biotechnol Adv.* 31: 266-273.

Auger C, Appanna V, Castonguay Z, Han S, and Appanna VD (2012). A facile electrophoretic technique to monitor phosphoenolpyruvate-dependent kinases. Electrophoresis 33: 1095-1101.

Auger C, Lemire J, Cecchini D, Bignucolo A and Appanna VD (2011) The Metabolic Reprogramming Evoked by Nitrosative Stress Triggers the Anaerobic Utilization of Citrate in *Pseudomonas fluorescens*. PLoS One 6:e28469

Bignucolo A, Appanna VP, Thomas SC, Auger C, Han S, Omri A and Appanna VD(2013) Hydrogen peroxide stress a metabolic reprogramming in *Pseudomonas fluorescens*: Enhanced production of pyruvate. J Biotech. 167: 309-315

Bradford MB (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254

- Charoo NA, Shamsheer AAA, Lian LY, Abrahamsson B, Cristofolletti R, Groot DW, Kopp S, Langguth P, Polli J, Shah VP, and Dressman J (2013) Biowaiver Monograph for Immediate-Release Solid Oral Dosage Forms: Bisoprolol Fumarate. *J Pharma Sci.* 103: 378-391
- Chénier D, Bériault R, Mailloux R, Baquie M, Abramia G, Lemire J and Appanna VD (2008) Metabolic adaptation in *Pseudomonas fluorescens* evoked by aluminum and gallium toxicity: Involvement of fumarase C and NADH oxidase. *App Environ Micro.* 74: 3977-84.
- Coppola D, Giordano D, Tianjero-Trejo Mariana, di Prisco G, Ascenzi P, Poole RK, and Verde C (2013) Antarctic bacterial haemoglobin and its role in the protection against nitrogen reactive species. *Biochem Biophys Acta.* 1834: 1923-1931.
- Couston V, Besterio S, Biran M, Diolez P, Bouchaud V, Voisin P, Michels PAM, Canioni P, Baltz T, and Bringaud F (2003). ATP generation in the *Trypanosoma brucei* procyclic form cytosolic substrate level phosphorylation is essential, but not oxidative phosphorylation. *J Biol Chem.* 278: 49625-49635
- Hall JW, and Ji Y (2013). Sensing and adapting to anaerobic conditions by *Staphylococcus aureus*. *Adv App Microbiol.* 84: 1-25.
- Han S, Auger C, Thomas SC, Beites CL and Appanna VD (2013). Mitochondrial biogenesis and energy production in differentiating stem cells: a functional metabolic study. *Cell Reprogram.* 16: 84-90.

- Hunt KA, Flynn JM, Naranjo B, Shikhare ID, and Gralnick JA (2010). Substrate-Level Phosphorylation Is the Primary Source of Energy Conservation during Anaerobic Respiration of *Shewanella oneidensis* Strain MR-1. *J Bacteriol.* 192: 3345-3351.
- Ingram-Smith C, Martin SR, and Smith KS (2006). Acetate kinase: not just a bacterial enzyme. *Trends Microbiol.* 14: 249-253.
- Kim D, Yu BJ, Kim JA, Lee Y, Choi S, and Kang S (2013). The acetylproteome of Gram positive model bacterium *Bacillus subtilis*. *Proteomics* 13: 1726-1736.
- Lemire J, Auger C, Bignucolo A, Appanna VP, and Appanna VD (2012). Metabolic Strategies Deployed by *Pseudomonas fluorescens* to Combat Metal Pollutants: Biotechnological Prospects in Current Research, Technology and Education topics in Applied Microbiology and Microbial Biotechnolog. *Formalex Publisher, A. Mendez-vilas (Ed.)* pp. 177-187.
- Lemire J, Mailloux R, Appanna VD (2008) Zinc toxicity alters mitochondrial metabolism and leads to decreased ATP production in hepatocytes. *J App Toxicol.* 28: 175–182.
- Li K, Pidatala RV, Shaik R, Datta R, and Ramakrishna W (2014). Integrated metabolomic and proteomic approaches dissect the effect of metal-resistant bacteria on maize biomass and copper uptake. *Environ Sci Technol.* 48: 1184-1193.
- Lien SK, Sletta H, Ellingsen TE, Valla S, Correa E, Goodacre R, Vernstad K, Borgos SEF, and Burheim P (2013). Investigating alginate production and carbon utilization in *Pseudomonas fluorescens* SBW25 using mass spectrometry-based metabolic profiling. *Metabolomics.* 9: 403-417.

- Lushchak OV, Piroddi M, Galli F, and Lushchak VI (2014). Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species. *Redox Rep.* 19:8-15.
- Ma Y, Guo C, Li H, and Peng X (2013). Low abundance of respiratory nitrate reductase is essential for *Escherichia coli* in resistance to aminoglycoside and cephalosporin. *J Proteomics.* 87: 78-88.
- Mailloux RJ, and Appanna VD (2007). Aluminum toxicity triggers the nuclear translocation of HIF-1alpha and promotes anaerobiosis in hepatocytes. *Toxicol In Vitro.* 21: 16–24
- Mailloux RJ, Lemire J and Appanna VD (2011). Metabolic Networks to Combat Oxidative Stress in *Pseudomonas fluorescens*. *Antonie van Leeuwenhoek* 99: 433-442
- Miranda KM, Espey MG, and Wink DA (2001). A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 5: 62–71.
- Poole RK (2005). Nitric oxide and nitrosative stress tolerance in bacteria. *Biochem Soc Trans.* 33: 176-180
- Quijano C, Alvarez B, Gatti RM, Augusto O, and Radi R (1997). Pathways of peroxynitrite oxidation of thiol groups. *Biochem J.* 322: 167-173.
- Schagger H, and von Jagow G (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal Biochem.* 199: 223-231.

- Singh R, Chenier D, Beriault R, Mailloux R, Hamel RD, and Appanna VD (2005). Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate producing enzymes. *J Biochem Biophys Methods*. 64: 189–199.
- Steinsiek S, Stagge S, Battenbrock K (2014). Analysis of *Escherichia coli* Mutants with a Linear Respiratory Chain. *PloS One* 9: 1-15.
- Watanabe S, Zimmerman M, Goodwin MB, Sauer U, Barry CE, Boshoff HI (2011). Fumarate reductase activity maintains an energized membrane in anerobic *Mycobacterium tuberculosis*. *PloS One* 7: 1-15.
- Zielonka J, Zielonka M, Sikora A, Adamus J, Joseph J, Hardy M, Ouari O, Dranka BP, and Kalyanarama B (2012). Global Profiling of Reactive Oxygen and Nitrogen Species in Biological Systems: high-throughput real-time analyses. *J Biol Chem*. 287: 2984-2995.

2.6: Figures

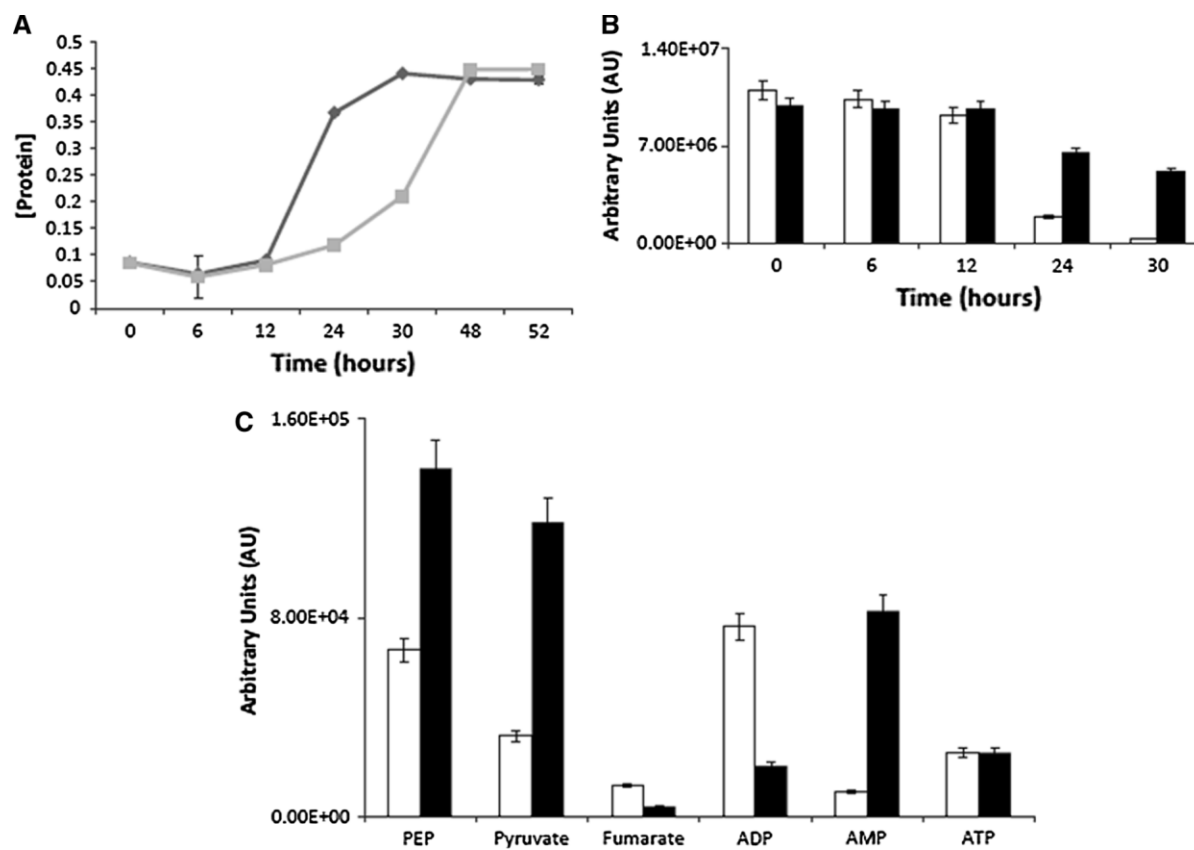


Figure 1: A Effects of nitrosative stress on *Pseudomonas fluorescens*, A Growth profiles of control and RNS-stressed cultures

B Fumarate consumption in control and stressed cultures as measured with HPLC at 236 nm. n = 3. (SD \pm), C Select metabolites and nucleotides in the soluble cell free extract (sCFE).

□: Control, ■: Stressed cultures (n = 3; SD \pm)

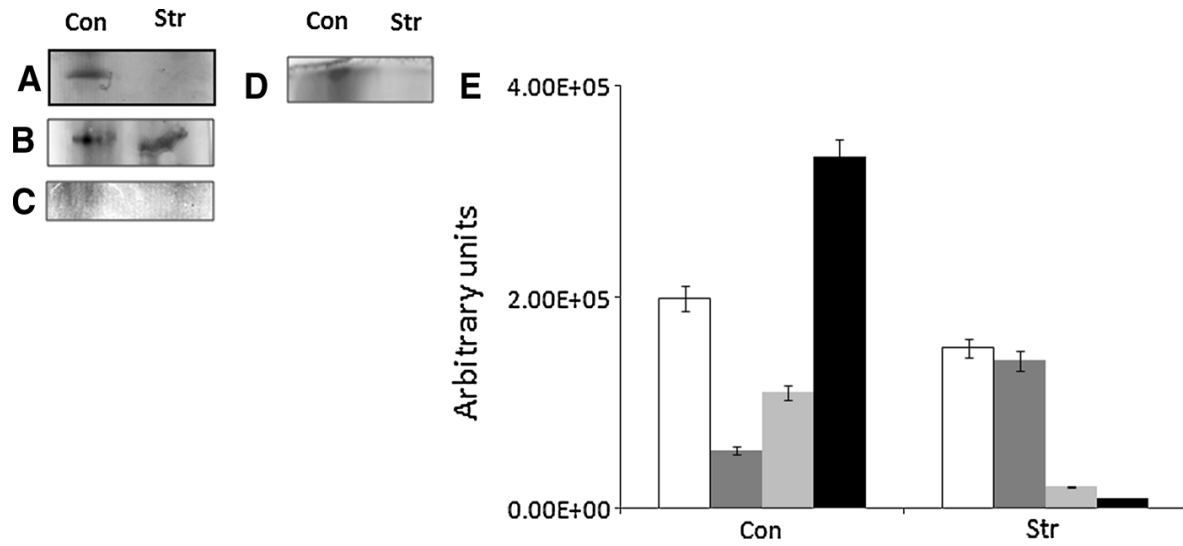


Figure 2: Enzymatic activity of select TCA cycle and ETC enzymes: A ICDH-NAD⁺, B MDH, C Complex II, D Complex IV (Con: Control, Str: Stress): E Fumarate metabolism in *P. fluorescens* (n = 3). □: ATP without KCN, ■: ATP with KCN, ▨: Fumarate consumption without KCN, ▩: Fumarate consumption with KCN. Con: Control, Str: Stress

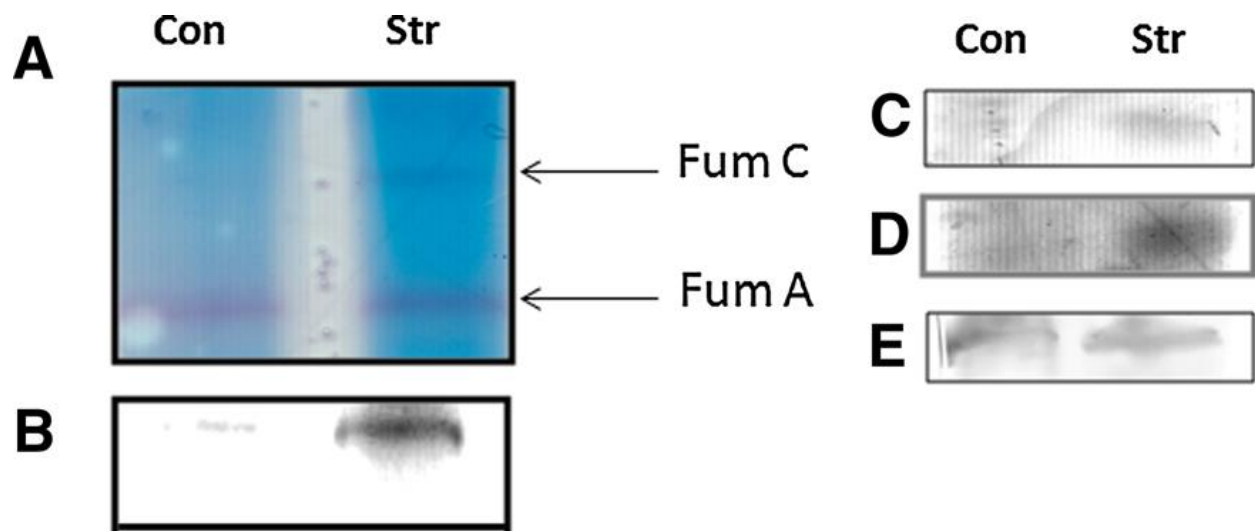


Figure 3: Enzymes involved in fumarate metabolism; A Fumarase isoforms Fum A and Fum C, B Fumarate reductase (FRD), C Phosphoenolpyruvate carboxylase (PEPC), D Phosphoenolpyruvate dikinase (PPDK), E Pyruvate Carboxylase (PC).

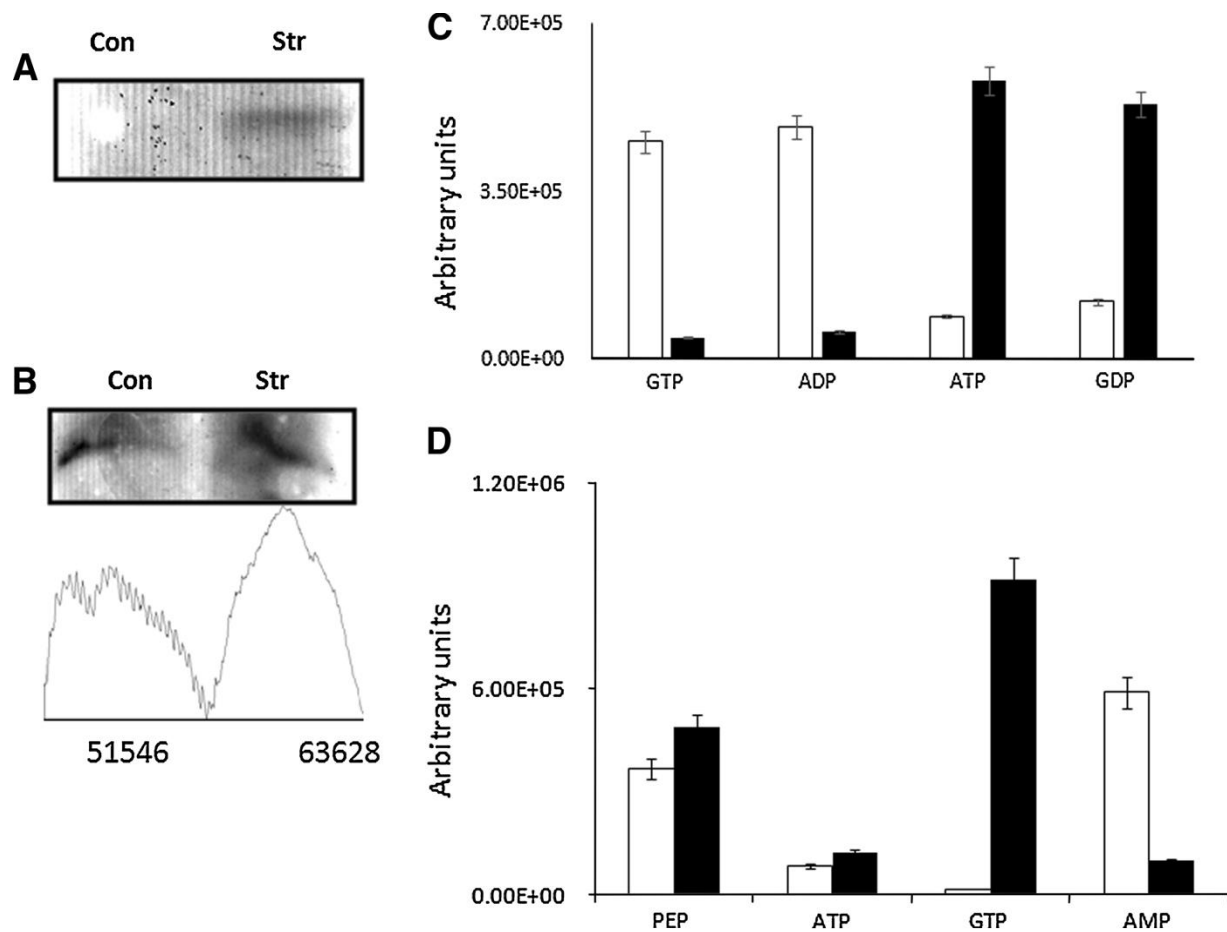


Figure 4: A Adenylate Kinase activity, B NDPK activity, and densitometric readings for in gel activity (n = 3) (Con: Control, Str: Stress), C The NDPK activity bands were incubated with ADP and GTP, note the production of ATP and GDP (□: Control, ■: Stress) n = 3. D Nucleoside triphosphate production by CFE in the presence of oxaloacetate, AMP, PP_i. (Con: Control, Str: Stress) n = 3 (SD AU ±) (□: Control, ■: Stress)

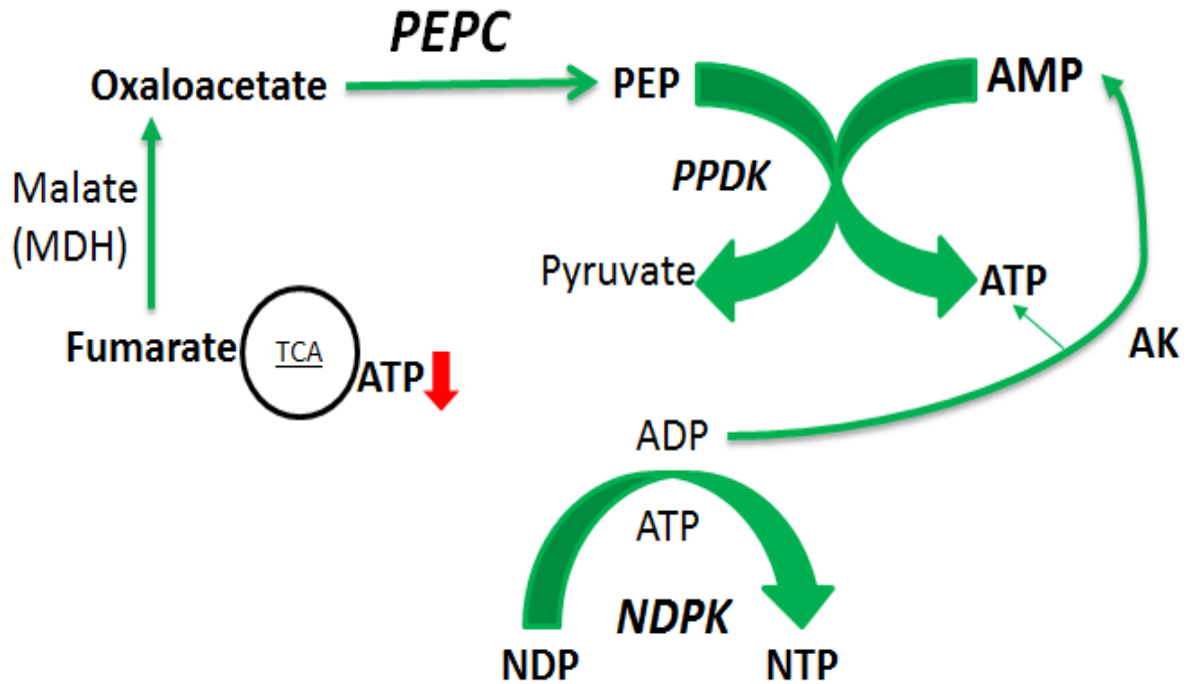


Figure 5: An alternate ATP-producing machinery in *P. fluorescens* exposed to nitrosative stress. Fumarate and AMP are key ingredients in this process.

Experiment	Control	Stress
Nitrate Reductase activity (AU) ^a	3597 ± 58	46501 ± 49
Nitrite Reductase activity (AU) ^a	2113 ± 62	37632 ± 97
Griess assay (NO ₂ ⁻)	2.10 µM ± 0.63 µM	102 µ ± 15 µM
Griess assay (NO ₃ ⁻)	2.78 µM ± 0.71 µM	113 µM ± 22 µM
ICDH-NAD ⁺ activity ^b (nmol/min mg/protein NADH)	106.67 ± 10.4	33.55 ± 2.1
MDH activity ^b (nmol/min mg/protein NADH)	60.43 ± 5.5	56.13 ± 3.2

Table 1: Activities of some enzymes, and nitrate and nitrite levels in control and stressed cultures.

Chapter 3: Conclusion and Future Perspectives

The findings in this project revealed that *P. fluorescens* was able to proliferate despite the negative impact of nitrosative stress on the growth rate. Although the RNS-challenged microbe multiplied slower than the control cultures, the cellular yield at stationary phase of growth was relatively similar. Enzymes such as nitrite reductase, nitrate reductase, and GSNOR were upregulated to detoxify the injurious effects of the RNS. There was a marked reduction in enzymes of the TCA cycle and ETC. These metabolic networks are critical for the generation of ATP in an O₂-dependent manner. Despite the drastic reduction of oxidative phosphorylation, *P. fluorescens* did thrive in the presence of RNS. To achieve this, a unique metabolic module was elaborated in an effort to generate ATP via substrate level phosphorylation (SLP). The increase activities of PEPC and PPDK help produce the high-energy PEP that was tapped to produce ATP. In this instance as AMP was phosphorylated to ATP, there was a net gain in energy as opposed to the traditional glycolytic system.

As Fe-S clusters in the widely occurring fumarase (FUM) are severely affected by RNS (Abe et al 2007), *P. fluorescens* evoked the synthesis of fumarase C (FUM C), an Fe-independent isomer that is known to be resistant to oxidative and nitrosative stress. The metabolism of fumarate was promoted by the increased activity of fumarate reductase (FRD), an enzyme that was barely discernable in the control cultures. Hence, the tandem of FUM C and FRD enabled the microbe to degrade fumarate, the sole carbon source in this defined medium. While the former mediates the formation of malate, the latter produces succinate. These two metabolites subsequently fuel the production of energy and enable the microbial system to proliferate.

An intricate phosotransfer system provided an effective vehicle to produce high energy phosphates and AMP. The latter is an essential ingredient that propels the PPDK-ATP generating system. Thus, PEPC, PPDK, AK, and NDPK worked in a collaborative fashion. This meant that the ATP-machinery did not necessitate the presence of O₂. This metabolic reconfiguration allowed *P. fluorescens* to neutralize the toxic influence of RNS, an attribute that numerous infectious organisms are known to invoke in an effort to invade various host-defense mechanisms (Fig. 3.1).

The enhanced activity of PEPC provides a very effective route to generate PEP, the high energy phosphate that was eventually tapped into ATP. The presence of PPDK mediated the conversion of PEP and AMP into ATP, a process more potent than the reaction mediated by pyruvate kinase (PK). The latter utilizes ADP to synthesize ATP from PEP (Anastasiou et al 2011). The upregulation in activities of AK helped supply adequate AMP with the concomitant formation of ATP, while the increased presence of NDPK ensured a pathway to transfer the high energy phosphate from ATP to other nucleoside diphosphate (NDP).

Although the nitrite and nitrate reductase aided in the detoxification of RNS into less harmful nitrite and nitrate, the increased production of pyruvate in the cultures exposed to nitrosative stress may also be an important tool in combatting RNS. This keto acid is known to neutralize RNS and ROS with the subsequent formation of acetate. The significance of observation needs to be further explored, such as possible antioxidant studies. The metabolic strategy uncovered in this thesis would have the added benefit of both providing a route to effective ATP-synthesis and the generation of pyruvate, a potent anti-oxidant. It is for these reasons that these two target enzymes provide an excellent therapeutic cue against RNS-resistant

microbes. Additionally studies can also be performed on other strains of *P. fluorescens* to see if other adaptations might be present.

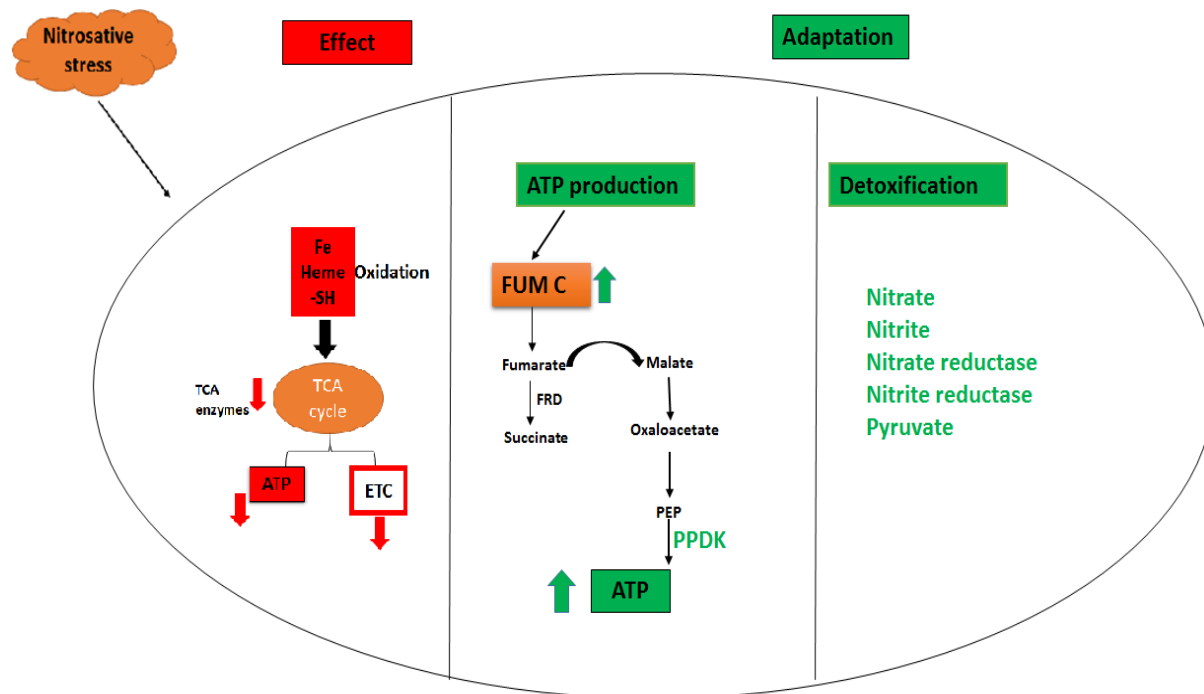


Figure 3.1: Summary of key findings on fumarate metabolism in *P. fluorescens* subjected to RNS stress. Note the novel ATP-producing mechanism independent of O_2 .

Fumarate metabolism is also of great interest as it can provide easy access to value-added products such as malate and succinate. Succinate has a variety of commercial applications in the pharmaceutical, plastic, and food industries. The discovery of the increase activity of the enzyme FRD may be investigated in an effort to unearth an easy route to the production of succinate. This enzyme, or the whole RNS-stressed *P. fluorescens* may be immobilized on a solid surface in an effort to provide succinate (Fig 3.2). The addition of fumarate or other renewable metabolites can provide a profitable means of producing succinate devoid of any contaminants.

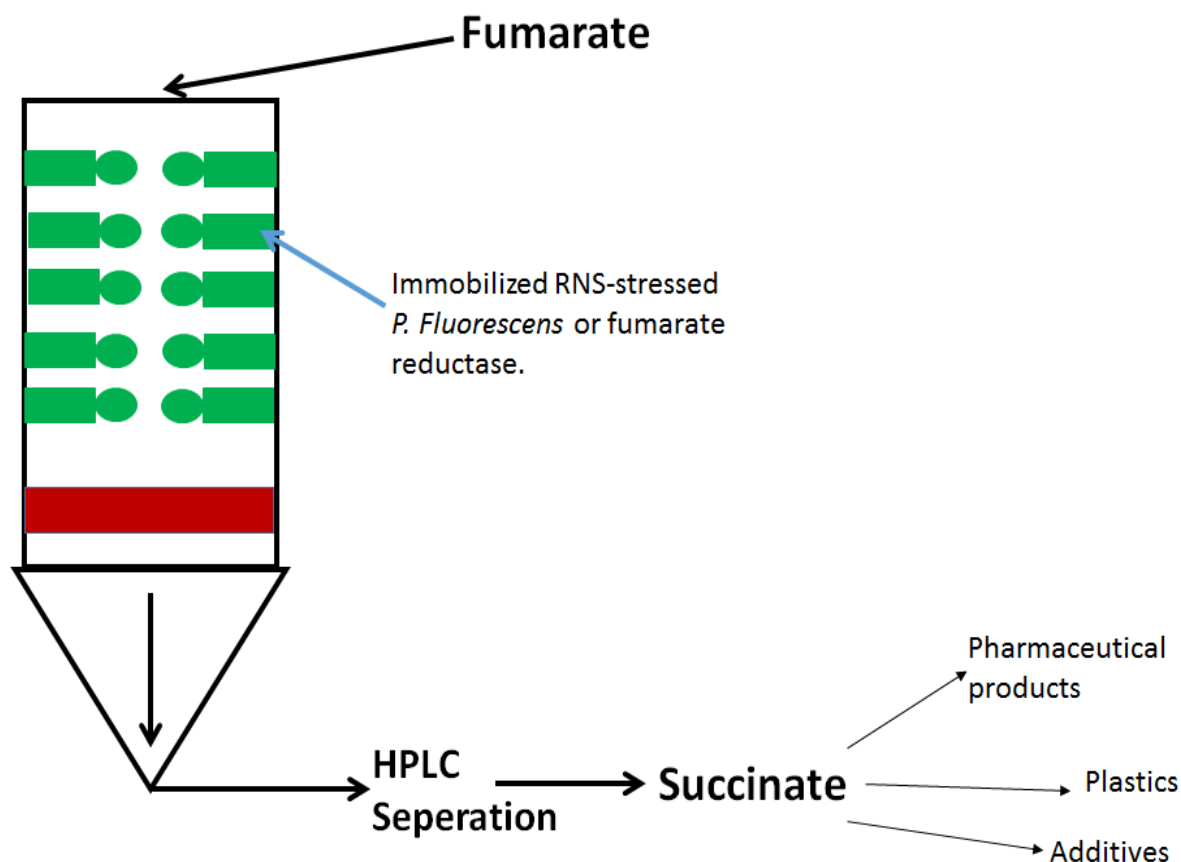


Figure 3.2: Outline of potential bioreactor for succinate production using fumarate. (Note the use of HPLC to separate metabolites and thus purify succinate for industrial applications).

This work has helped unravel a unique ATP-generating machinery that utilizes PEPC and PPDK as its key components and an elaborate network of phosphotransfer enzymes to mediate the transfer of High energy phosphate. These can be possible targets in curtailing RNS-resistant bacteria. Furthermore, RNS-tolerant *P. fluorescens* and/or its components can be tailored to produce succinate, an important commercial commodity.

Chapter 4: General Bibliography

Abe T et al (2007). Anaerobic elemental sulfur reduction by fungus *Fusarium oxysporum*. Bioscience, Biotechnology, and Biochemistry. 71: 2402-2407.

Agarwal R et al (2012). Magnesium deficiency: Does it have a role to play in cataractogenesis? Experimental Eye Research. 101: 82-89.

Akram M (2013). Mini-review on glycolysis and cancer. Journal of Cancer Education. 28(3): 454-457.

Alvarez CE et al (2013). Kinetics and functional diversity among the five members of the NADP-malic enzyme family from *Zea mays*, a C₄ species. Photosynthesis Research. 115(1): 65-80.

Bailey-Serres J et al (2012). Making sense of low oxygen sensing. Trends in Plant Science. 17(3): 129-138.

Baily CN et al (2011). Inhibition of mitochondrial respiration by phosphoenolpyruvate. Archives of Biochemistry and Biophysics. 514(1): 68-74.

Belenchiev IF et al (2012). The neuroprotective activity of tamoxifen and tibolone during glutathione depletion in vitro. Neurochemical Journal. 6(3): 202-212.

Besterio S et al (2002). Succinate secreted by *Trypanosoma brucei* is produced by a novel and unique glycosomal enzyme, NADH-dependent fumarate reductase. Journal of Biological Chemistry. 277: 38001-38012.

- Biegel E et al (2011). Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. *Cellular and Molecular Life Sciences*. 8(4): 613-634.
- Bourret TJ et al (2011). Nitrosative damage to free and zinc-bound cysteine thiols underlies nitric oxide toxicity in wild-type *Borrelia burgdorferi*. *Molecular Microbiology*. 81(1): 259-273.
- Brigaud F, Riviere L and Coustou V (2006). Energy metabolism of trypanosomatids: adaptation to available carbon sources. *Molecular and Biochemical Parasitology*. 149: 1-6.
- Carraro M et al (2014). Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition. *Journal of Biological Chemistry*. 289: 15980-15985.
- Celedon JM and Cline K (2013). Intra-plastid protein trafficking: How plant cells adapted prokaryotic mechanisms to the eukaryotic condition. *BBA: Molecular Cell Research*. 1833(2): 341-361.
- Chabriere E et al (1999). Crystal structures of key anaerobic enzyme pyruvateferredoxin oxidoreductase, free and in complex with pyruvate. *Natural Structure Biology*. 6: 182-190.
- Corpas FJ and Barroso (2013). Nitro-oxidative stress vs oxidative or nitrosative stress in higher plants. *New Phytologist*. 199(3): 633-635.
- Dalbey RE, Wang P and Kuhn A (2011). Assembly of bacterial inner membrane proteins. *Annual Review of Biochemistry*. 80: 161-187

- Deramchia K et al (2014). Contribution of pyruvate phosphate dikinase in the maintenance of the glycosomal ATP/ADP balance in the *Trypanosoma brucei* procyclic form. *Journal of Biological Chemistry*. 1-28.
- Fernie AR, Fernando C and Sweetlove LJ (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology*. 7(3): 254-261.
- Flamholz A et al (2013). Glycolytic strategy as a tradeoff between energy yield and protein cost. *Proceedings of National Academy of Sciences*. 110(24): 10039-10044.
- Graves DB (2012). The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology. *Journal of Physics: Applied Physics*. 45(26): 1-10
- Grochowski LL et al (2012). A new class of adenylate kinase in methanogens is related to uridylylate kinase. *Archives of Microbiology*. 194 (2): 141-145.
- Haq R et al (2013). Oncogenic BRAF Regulates Oxidative Metabolism via PGC1 α and MITF. *Cancer Cell*. 23(3): 302-315.
- Hayashi M et al (2012). Oxidative stress in developmental brain disorders. *Neurodegenerative Disease*. 724: 278-290.
- Heiden MG et al (2010). Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science*. 329(5998): 1492-1499.
- Hu Z et al (2013). Protection of cells from nitric oxide-mediated apoptotic death by glutathione C₆₀ derivative. *Cell Biology International*. 36(7): 677-681.

- Hunt KA et al (2010). Substrate-level phosphorylation Is the primary source of energy conservation during anaerobic respiration of *Shewanella oneidensis* strain MR-1. Journal of Bacteriology. 192(13): 3345-3351.
- Jiang S et al (2012). A novel miR-155/miR-143 cascade controls glycolysis by regulating *hexokinase 2* in breast cancer cells. EMBO Journal. 31(8): 1985-1998.
- Liu X, Sheng J and Curtiss III R (2011). Fatty acid production in genetically modified cyanobacteria. Proceedings of the National Academy of Sciences. 108 (17): 6899-6904.
- Luo et al (2010). Biorefining of lignocellulosic feedstock – Technical, economic and environmental considerations. Bioresource Technology. 101(13): 5023-5032.
- Lushchak VI (2011). Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. Comparative Biochemistry and Physiology: Toxicology & Pharmacology. 153(2): 175-190.
- Michaelson LP et al (2013). ROS and RNS signaling in skeletal muscle: critical signals and therapeutic targets. Annual Review of Nursing Research. 31(1): 367-387.
- Mishra P et al (2014). Proteolytic cleavage of opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. Cell Metabolism. 19(4): 630-641.
- McKinlay JB et al (2010). A genomic perspective on the potential of *Actinobacillus succinogenes* for industrial succinate production. BMC Genomics. 11:680.
- Murphy MP et al (2011). Unraveling the biological roles of reactive oxygen species. Cell Metabolism. 13(4): 361-366.

- Naik E and Dixit VM (2011). Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *Journal of Experimental Medicine*. 208(3): 417-420.
- Nicholson JK et al (2012). Host-gut microbiota metabolic interactions. *Science*. 336: 1262-1267.
- Omri A et al (2002). Enhanced activity of liposomal polymyxin B against *Pseudomonas aeruginosa* in a rat model of lung Infection. *Biochemical Pharmacology*. 64(9): 1407-1413.
- Picard T et al (2006). Protective and Inhibitory effects of various types of amphipols on Ca^{2+} ATPase for sarcoplasmic reticulum: a comparative study. *Biochemistry*. 45(6): 1861-1869.
- Pike LS et al (2011). Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *BBA: Bioenergetics*. 1807(6): 726-734.
- Raimundo N et al (2011). Revisiting the TCA cycle: signaling to tumor formation. *Trends in Molecular Medicine*. 17(11): 641-649.
- Reddy GK and Wendisch VF (2014). Characterization of 3-phosphoglycerate kinase from *Corynebacterium glutamicum* and its impact on amino acid production. *BMC Microbiology*. 14:54.
- del Rio LA (2011). Peroxisomes as a cellular source of reactive nitrogen species signal molecules. *Archives of Biochemistry and Biophysics*. 506(1): 1-11.

- Ritov VB et al (2010). Deficiency of electron transport chain in human skeletal muscle mitochondria in type-2 diabetes mellitus and obesity. *Endocrinology and Metabolism*. 298(1): 49-58.
- Salvemini D et al (2011). Roles of reactive oxygen and nitrogen species in pain, *Free Radical Biology and Medicine*. 51(5): 951-966.
- Satapati S et al (2012). Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *Journal of Lipid Research*. 53: 1080-1092.
- Selvilla E et al (2013). The *Pseudomonas putida* HskA hybrid sensor kinase responds to redox signals and contributes to the adaptation of the electron transport chain composition in response to oxygen availability. *Environmental Microbiology Reports*. 5(6): 825-834.
- Selyunin AS et al (2011). The assembly of a GTPase-kinase signalling complex by bacterial catalytic scaffold. *Nature*. 469(7328): 107-111.
- Sharma P et al (2012). Uncoupling of substrate-level phosphorylation in *Escherichia coli* during glucose-limited growth. *Applied and Environmental Microbiology*. 78(19): 6908-6913.
- Suntres ZE et al (2002). *Pseudomonas aeruginosa*-induced lung injury: Role of oxidative stress. *Microbial Pathogenesis* . 32: 27-34.
- Skulachev VP et al (2013). Principles of Bioenergetics. 139-156.
- Thakker C et al (2011). Succinate production in *Escherichia coli*. *Biotechnology Journal*. 7(2): 213-224.

- Trapani S et al (2001). Crystal structure of the dimeric phosphoenolpyruvate carboxykinase (PEPCK) from *Trypanosoma cruzi* at 2 Å resolution. *Journal of Molecular Biology*. 313: 1059-1072.
- Vitetta L and Linnane AW (2014). Endocellular regulation by free radicals and hydrogen peroxide: key determinants of the inflammatory response. *Inflammopharmacology*. 22(2): 69-72.
- Voskull MI et al (2011). The response of *Mycobacterium tuberculosis* to reactive oxygen and reactive nitrogen species. 2: 1-12
- Wang F et al (2013). Arsenite-induced ROS/RNS generation causes zinc loss and inhibits the activity of poly(ADP-ribose) polymerase-1. *Free Radical Biology and Medicine*. 61: 249-256.
- Williams BA et al (2008). Distinct localization patterns of two putative mitochondrial proteins in the microsporidian *Encephalitozoon cuniculi*. *Journal of Eukaryotic Microbiology*. 55: 131-133.
- Wyatt KH et al (2014). Effects of nutrient limitations on release and use of dissolved organic carbon from benthic algae in Lake Michigan. *Chicago Journals*. 33: 1-12.
- Yao Y et al (2011). Enhanced adenosine triphosphate production by *Saccharomyces cerevisiae* using an efficient energy regeneration system. *Korean Journal of Chemical Engineering*. 28(1): 178-183.